ANALYSIS OF GROWTH, PHENOTYPIC CHARACTERISTICS AND EXPRESSION OF STRO1 AND CD106, IN DENTAL PULP STEM CELLS AND STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH – A CELL CULTURE STUDY

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In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

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CERTIFICATE

This is to certify that this dissertation titled **"Analysis of Growth, Phenotypic characteristics and Expression of STRO1 and CD106, in Dental Pulp Stem Cells and Stem cells from Human Exfoliated Deciduous teeth – a cell culture study**" is a bonafide record of work done by **Soundarya S** under our guidance during her postgraduate study period between 2009-2012.

This dissertation is submitted to **THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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All praise and glory to GOD Almighty for nothing could have been done without

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Grace

"He has made everything beautiful in its time"

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Introduction

Postnatal stem cells have been isolated from various tissues, including bone marrow, neural tissue, skin, retina, dental epithelium, dental pulp, periodontal ligament dental follicle apical papilla, and adipose tissue. Previously, it was generally accepted that the differentiation potential of postnatal stem cells was lineage restricted but recent research has proved that post natal stem cells have the potential to overcome the lineage restrictions and thus possess the potential to differentiate into specific cells of tissues beyond the cell of origin [1, 2, 3].

Stem cells reside in defined microenvironments termed niches. A niche is considered to be a subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo*. Researchers in different parts of the world have identified various stem cell niches in the human body. The dental pulp that is contained within the closed compartment of pulp chamber serves to be an affluent source of stem cells as "sealed niches" [4]. They are dormant but have the potential to express stem cell features. Such stem cells from pulp of permanent teeth and deciduous teeth are called Dental Pulp Stem Cells (DPSCs) and Stem cells from Human Exfoliated Deciduous teeth (SHED) respectively.[1, 9]

Both DPSCs and SHED are mesenchymal stem cells expressing characteristics similar to that of the Bone Marrow derived Mesenchymal Stem Cells (BMMSCs). Accumulated awareness of phenotypic features of BMMSCs has contributed appreciably to the isolation of recognized stem cells from the dental pulp of both permanent and deciduous teeth.

Stem cells have the property of continuous rejuvenation and expansion all through an individual's life but they reveal restricted proliferation and differentiation ability in an *ex-vivo* setting. Mesenchymal stem cell capability to expand is vastly unpredictable, even between two samples from the same patient. Early MSCs are pluripotent, while late MSCs had restricted differentiation capability.

Characterization of mesenchymal stem cells is a step towards understanding the applications of these cells therapeutically. Characterization can be done morphologically, immunologically or genetically. Morphological characterization involves the study of cell shape, size and growth characteristics.

The defining characteristics of stem cells are studied by utilising stem cell markers. The panel of antibodies include embryonal markers like the Oct-4, Nanog and Surface Specific Embryonal Antigen (SSEA) and cell surface markers like STRO1 and CD 106.

STRO1 is by far the best-known MSC marker. $STRO1^{+}$ positive DPSCs consist of at least three interrelated subpopulations including the progenitors of odontoblasts,osteoblasts, and chondrocytes, which can form dentin, bone, and cartilage tissues respectively. [5]

CD106, or VCAM-1 (Vascular Cell Adhesion Molecule-1), is expressed on blood vessel endothelial and adjoining cells, dependable with the fact due to the perivascular position of MSCs. CD106 singles out 1.4% of STRO1-positive cells. These cells increase the incidence of Colony Forming Unit of fibroblasts

(CFU –F) 1 in 3. All these cells are all high STRO1-expressing cells and are the only STRO1^{+} positive cells that form colonies and show stem cell characteristics such as multipotentiality, expression of telomerase, and high proliferation *in vitro*.[6] Thus STRO1 and CD106 combine to make good human MSC markers.[6]

The present study was done to isolate and expand the mesenchymal cell population from the dental pulp of both deciduous and permanent teeth, to analyse and compare their growth and phenotypic characteristics and also to standardize their method of molecular characterization using STRO1 and CD 106 monoclonal antibodies as an attempt to ascertain their feasibility for utility in therapeutic purposes.

Aims & Objectives

AIM

To isolate, culture and study the morphology of mesenchymal stem cells from the dental pulp of permanent teeth (DPSC) and exfoliated deciduous teeth (SHED), and compare their growth characteristics and their expression of STRO1 and VCAM (CD106) by immunophenotypic characterization.

OBJECTIVE

1. To isolate and culture mesenchymal cells from permanent teeth (DPSCs) and exfoliated deciduous teeth (SHED) using enzyme disaggregation technique.
2. To compare the phenotypic and growth characteristics of cells isolated from the dental pulp of permanent teeth (DPSCs) and exfoliated deciduous teeth (SHED).
3. To identify the stem cells within the mesenchymal cells isolated from the pulp of permanent and deciduous teeth using mesenchymal stem cell markers STRO1 and VCAM (CD 106).

HYPOTHESIS

Null hypothesis

There exists no difference in growth characteristics, phenotype and expression of stem cell markers STRO1 and CD 106 (VCAM – Vascular Cell Adhesion Molecule) between the Dental Pulp Stem Cells (DPSCs) and Stem cells from Human Exfoliated Deciduous teeth (SHED).

Alternate hypothesis

There exists a difference in growth characteristics, phenotype and expression of stem cell markers STRO1 and CD 106 (VCAM – Vascular Cell Adhesion Molecule) between the DPSCs and SHED.

Materials & Methods

MATERIALS FOR TISSUE CULTURE:

Reagents

Growth medium-

1. Mesenchymal Stem Cell Medium (MSC Medium) - α- modified minimal essential medium (α-MEM) (Invitrogen ^{TM})
2. Fetal Bovine Serum (Invitrogen ^{TM})
3. Antibiotics-
    a. Penicillin-100 IU/ml.
    b. Streptomycin-100µg/ml.
4. Dulbecco's Phosphate Buffered Saline (Potassium chloride-0.2g/l, Potassium phosphate monobasic-0.2g/l,
5. Sodium chloride-8g/l, Sodium phosphate dibasic-1.15g/l)
6. Distilled water.
7. De-ionized water.
8. Collagenase (type I, filtered) (Hi Media ^{TM})
9. Collagenase. (C0130 – Sigma ^{TM})
10. Dispase (neutral protease, grade II) (Roche ^{TM})
11. Trypsin 1:125. (Tissue culture grade, Hi media ^{TM})
12. Ethylene-di-amine-tetra-acetic acid. (Hi Media ^{TM})

Equipment

1. Culture dishes. (Tarsons ^{TM})
2. 24-well plates. (Cell star ^{TM})
3. Plastic micropipettes
4. Micro pipette tips (Tarsons ^{TM})
5. Glass pipettes.

6. BP blade no. 15 and BP handle no. 4
7. Centrifuge tubes. (Tarsons ™)
8. Leak-proof screw-cap vials.
9. Scott Duran bottles.
10. Reusable bottle top filter (Tarsons ™)
11. Laminar flow cabinets.
12. Carbon dioxide incubator. (Thermo electron Corporation. Forma series II water jacketed-HEPA class 100)
13. Phase contrast microscope. (Olympus CKX41 ™)
14. Digital camera. (Kodak AF3X, 8.2 mega pixels, 3x optical zoom)
15. Improved Neubauer counting chamber.
16. Laboratory centrifuge. (R-86 Remi ™)
17. Cyclomixer. (C101 Remi ™)
18. Electronic balance. (Dhona 200D ™)
19. Prabivac vacuum pump.
20. Cellulose acetate filter (pore size 0.2µm)
21. Autoclave
22. Hot air oven
23. Micromotor (Marathon ™)
24. Contra – angled hand piece (NSK ™)
25. Carborundum discs
26. Endodontic files (Mani dia burs ™)
27. Chisel
28. Mallet
29. Wooden blocks

30. Enamel tray

Materials for immuncytochemistry

Reagents

1. Antibodies (Abcam ™)
    a. Mouse monoclonal [7i35] to STRO1 **[Annexure – VIII]**
    b. Mouse monoclonal [1G11B1] to VCAM **[Annexure – IX]**
    c. Rabbit polyclonal secondary antibody to mouse IgG – H&L (HRP) **[Annexure – X]**
2. Bovine serum albumin (Himedia ™)
3. Tween 20 (Himedia ™)
4. Sodium azide (Loba chemie ™)
5. Glycerol for molecular biology (SRL ™)
6. Phosphate buffered saline# (sodium chloride 7.714g/l, dipotassium hydrogen ortho phosphate 1.496g/l , potassium dihydrogen orthophosphate .204g/l)
7. Acetone (Merck ™)
8. APES (3-aminopropyl-triethoxy-silane)
9. Paraformaldehyde (chenchems)
10. Hydrochloric acid (Merck ™)
11. Sodium hydroxide
12. DPX (distrene , dibutyl phthalate, xylene)

Equipments

1. Glass slides
2. Micro centrifuge tubes (Tarsons™)
3. Cryo boxes (Tarsons™)

4. Couplin jars
5. Humidified chamber.
6. Electronic timer
7. Cover slips
8. Light microscope.

IRB approval

The study was carried out in Ragas dental college after approval was obtained from the Institutional Review Board. Informed consent was obtained from patients above the age of eighteen years and from the parents of children for the collection of teeth. **[Annexure – XI]**

Sample considered

We successfully isolated 5 DPSC from 12 samples of permanent teeth and 6 SHED from 9 deciduous teeth. **[Annexure –I, II]**

Transportation of tissue to laboratory for culturing

Teeth extracted under sterile condition was transferred to serum-free α-Minimal Essential Medium(α-MEM), with added antibiotics (Penicillin-100 IU, Streptomycin-100µg/ml), at a pH of 7.2 to 7.4 and maintained at 4°C with the help of ice-packs. They were transported in leak-proof, sterilized culture vials.

Protocol for isolation of dental pulp:

a. Tooth surface was cleaned well by washing thrice with Dulbecco's Phosphate Buffered Saline (D-PBS).
b. Grooves were placed around the cemento-enamel junction with a carborundum disc and ice-cold D-PBS irrigation to avoid heating while cutting.
c. Tooth was split with chisel and mallet to expose the pulp chamber.

d. The pulp tissue was obtained from the pulp chamber with the help of forceps and spoon excavator and put into 2ml of Mesenchymal Stem Cell (MSC) medium containing 1x antibiotics (Penicillin-100 IU, Streptomycin-100µg/ml) in leak proof screw cap vials.

Protocol for primary culture of dental pulp cells:

a. The dental pulp tissue was minced into tiny pieces with a surgical blade.

b. The tissue was immersed into a mixed collagenase (2mg) and dispase (1mg) solution in 1ml serum containing mesenchymal stem cell medium (α-mem) in centrifuge tubes.

c. It was incubated at 37^0C for overnight.

d. Cells were centrifuged at 2400rpm for 5 minutes.

e. The supernatant was removed and the pellet re-suspended with MSC medium.

f. The cells were cultured in MSC medium at 37^0C and 5 % CO_2 in the incubator.

Forty-eight hours after the cell isolation, the culture media was discarded and fresh media added to the Petri dish. Media change was repeated every third day until cell confluence was reached.

Protocol for Subculture:

a. The culture was examined carefully for signs of deterioration or contamination.

b. The media was discarded from the plate.

c. Two washes with 2ml D-PBS was done to remove any residual serum.

d. 1ml trypsin 0.25% with EDTA 0.05% was added to the Petri dish (60mm diameter).

e. The monolayer was checked under the microscope to see whether the cells were rounding.

f. The plate was tapped at the bottom until all the cells were detached.

g. Cells suspended in trypsin was collected in a centrifuge tube and centrifuged at 2400 rpm for 3 minutes.

h. Supernatant obtained after centrifugation was discarded. To the remaining cell pellet, the medium was added and cells were dispersed by repeated pipetting.

i. The cells were counted in a haemocytometer.

j. The cell suspension was diluted to appropriate seeding concentration by adding adequate volume of medium in a culture plate.

The plates were closed and returned to the incubator.

Cell culture studies:

i. Estimation of growth curves and its derivatives in cell lines

1. Cells from each culture were inoculated at 1.2 x 10^4 cells /ml/well on 24 welled plates.
2. After overnight attachment, cells from 3 randomly selected wells were trypsinized and counted using a haemocytometer.

3. The medium was changed on 3^{rd} and 6^{th} day.

4. The count was repeated every 24 hours for 8 days.

5. Cells from each well were counted thrice to avoid error.

6. The averages of daily cell counts of each well were used to plot the growth curve.

7. The seeding cell count and cell count one the first day ie, 12 hours of seeding is used to estimate the seeding efficiency in percentage by using the equation

$$\frac{\text{Cell count/well/ml after 12 hours X 100}}{\text{Seeding cell count/well/ml}}$$

8. The population doubling time was calculated from the slope of the growth curve at its log phase.

ii. Observation and recording of the F1, F2, F3 fibroblast subpopulation in cell lines :

1. All cell lines between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml.
2. Using a phase contrast microscope at 20x magnification we observed and counted the cells for 8 consecutive days and classified them according to their morphology into F_1, F_2 and F_3 fibroblasts according to the description by Mollenhauer and Bayreuther in 1986.[26]
3. Thirty cells were randomly recorded in each tissue culture plate giving a total of 90 cells per cell line.

iii. Observation and recording of the F1, F2, F3, F4, F5, F6, and F7 and mitotic and post-mitotic fibroblast subpopulation in cell lines:

1. All cell lines between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml.
2. Using a phase contrast microscope at 20x magnification we observed and counted the cells for 8 consecutive days and classified them according to their morphology into mitotic (F1,F2,F3) and post-mitotic (F4,F5,F6 and F7) fibroblasts according to the description by Bayreuther *et al* in 1988. [27]

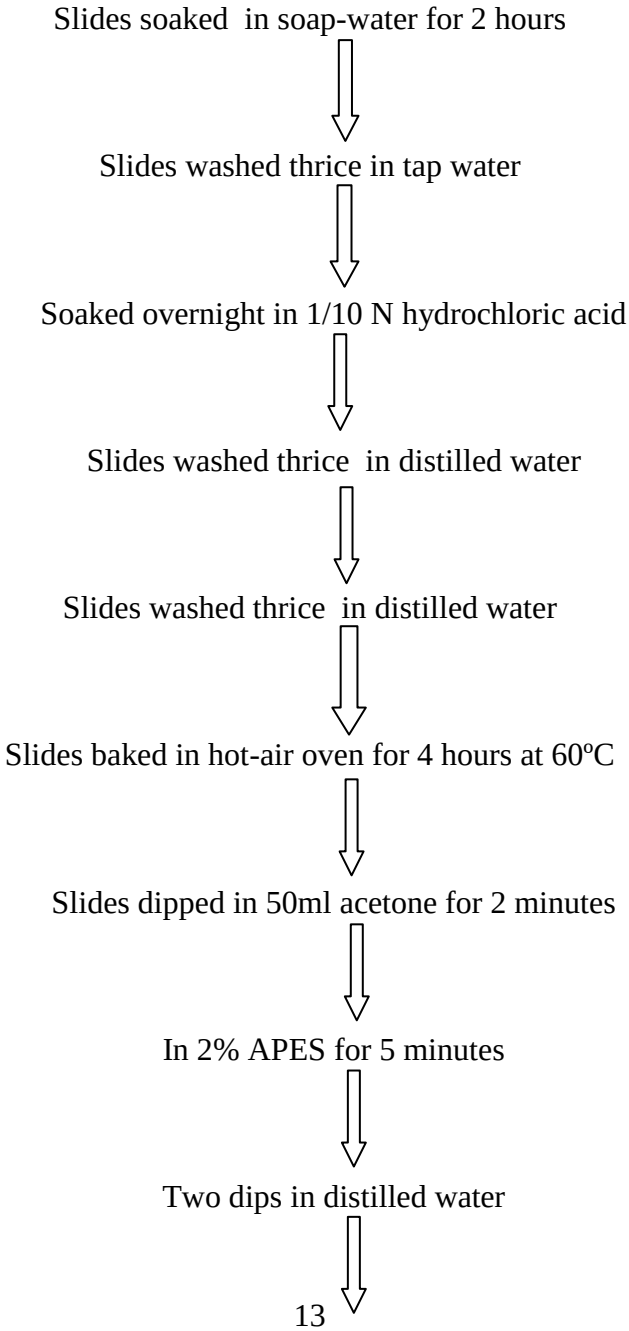
3. Thirty cells were randomly recorded in each tissue culture plate giving a total of 90 cells per cell line.

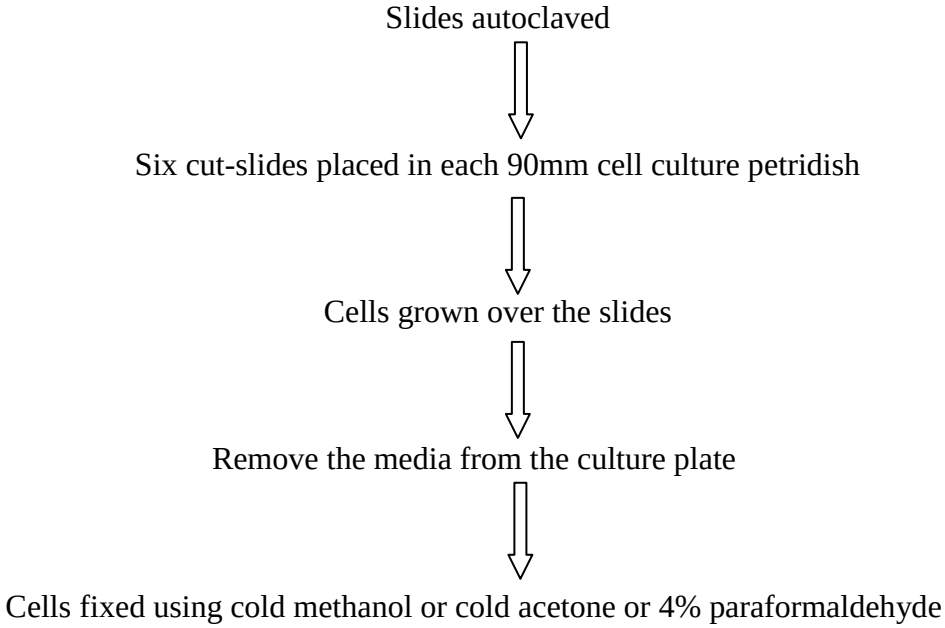
Protocol for immunocytochemistry

Sample consideration for immunocytochemistry

Cells grown on APES coated slides were considered for immuno cytochemistry.

Protocol for growing and fixing of cells on APES coated slides




Slides autoclaved
Six cut-slides placed in each 90mm cell culture petridish
Cells grown over the slides
Remove the media from the culture plate
Cells fixed using cold methanol or cold acetone or 4% paraformaldehyde

Protocol for immunocytochemistry

Wash thrice with PBS[#] (5 minutes each)

⇩

Incubate cells with 1% BSA in PBS [#] T for 30 minutes

⇩

Incubate cells with diluted primary antibody in a humidified chamber for 1 hour at room temperature or overnight at 4°C.

⇩

Decant the solution and wash the cells three times in PBS[#] (5 minutes each)

⇩

Incubate cells with the secondary antibody in 1% BSA for 1 hour at room temperature in dark

⇩

Decant the secondary antibody solution and wash three times with PBS[#] (5 minutes each in dark)

⇩

Counter stain with Hematoxylin

⇩

Rinse with distilled water

⇩

Mount with DPX

As suggested by ABCAM in the data sheet ab7219 **[annexure IX]** we used lymphnodes as positive controls for the CD 106.

Osteosarcoma cell lines were used as positive control for STRO1.

(Adhikari S et al[34] V A Siclari and L Qin 2010[42])

Statistical Analysis

STATISTICAL ANALYSIS

Data analysis was done using SPSS (statistical package for social science) version 10.0.5.

Linear regression analysis was used to derive the slope from growth curves of each cell populations for determination of the population doubling time.

Mann-Whitney Test was done to compare the seeding efficiency and the population doubling time between the permanent and deciduous tooth derived cell populations.

Correlation coefficient were determined to compare the F1, F2 and F3 of each sample.

Correlation coefficient were determined to compare mitotic F1, F2, F3 and post mitotic F4, F5, F6, and F7 ratios among the permanent and deciduous tooth pulp derived cell populations.

Review of Literature

Stem cells are characterized by their self-renewal properties and by their capacity to generate differentiated cell lineages by two mechanisms as follows. [7]

a) obligatory asymmetric replication, in which, with each stem cell division, one of the daughter cells retain its self-renewing capacity while the other enters a differentiation pathway, and
b) stochastic differentiation, in which a stem cell population is maintained by the balance between stem cell divisions that generate, either two self-renewing stem cells or two cells that will differentiate.

IMPORTANT MILE STONES

- 1908 - The term "stem cell" was proposed for scientific use by the Russian histologist Alexander Maksimov (1874–1928) at congress of hematologic society in Berlin. It postulated existence of haematopoietic stem cells
- 1991 – Caplan's concept of mesenchymal stem cells from bone marrow.[8]
- 2000 – First successful isolation and characterization of dental pulp stem cells by **Gronthos S, Mankani M, Brahim J, et al in 2000** [9]
- 2003 - **Miura M, Gronthos S, Zhao M in 2003**[1] of NIH discovers new source of adult stem cells in children's primary teeth.

Classification of stem cells based on origin [10]:

1. Stem cells from embryos
    - a. embryonic stem cells (blastocyst)
    - b. embryonic germ cells (gonadal ridge)
2. Stem cells from the fetus
    - fetal stem cells (abortus – fetal tissue)
3. Stem cells from the umbilical cord (infant)

- a. umbilical cord blood stem cells (umbilical cord blood)
- b. umbilical cord matrix stem cells (Wharton's jelly)

4. Stem cells from the adult.
    - a.Hematopoietic stem cells (Bone marrow, Peripheral blood)
    - b. Mesenchymal stem cells (Bone marrow stroma, Dental pulp, adipose tissue)
    - c. Liver stem cells
    - d. Epidermal (skin , hair)stem cells
    - e. Neuronal stem cells
    - f. Eye stem cells
    - g.Gut stem cells
    - h. Pancreatic stem cells

Stem cells present in early embryonic stages are pluripotent and can generate all of the cell types found in adult organisms, whereas, adult stem cells exhibit a continuum of plasticity or multipotency. The mesenchymal stem cell (MSC) is one of the most interesting adult stem cell types. These cells are easily isolated, cultured, and manipulated *ex vivo.* MSCs exhibit great plasticity and harbour the potential for therapeutic applications, but these cells are poorly defined. This has led to heterogeneity of names and phenotypes ascribed by different groups to this cell population.[11]

Although various sources of multipotent mesenchymal stem cell are identified Bone marrow derived stem cells first described by Friedenstein et al. are still renowned as the gold standard criterion for comparing the different mesenchymal stem cells. The various sources of mesenchymal stem cells include

adipose tissue, peripheral blood, lung, heart. These diverse group have also shown potential for proliferation and differentiation into different cell types like osteogenic, chondrogenic, adipogenic, myogenic, neurogenic and tenogenic. [3]

With discovery of post natal dental pulp stem cells (DPSCs) by Gronthos et al 2000 [9], various other sources of mesenchymal stem cells are identified in dental tissues that includes the stem cells from deciduous teeth (SHED) by **Miura M, Gronthos S, Zhao M in 2003 [1]**, Periodontal ligament stem cells (PDLSCs) by Seo et al in 2004, Stem cells from apical follicle (SCAP) by Sonoyama et al in 2006, and the dental follicle precursor cells (DFPCs) by Morsczeck et al in 2005.

ISOLATION

DENTAL PULP STEM CELLS (DPSCs)

Gronthos S, Mankani M, Brahim J, et al in 2000 [9] first successfully isolated DPSCs from the pulp of impacted molars of human aged 19 -29 years. The teeth were collected and cut around the cementum-enamel junction by using sterilized dental fissure burs, to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root and then digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 hour at 37°C. Single-cell suspensions were obtained by passing the cells through a 70-mm strainer. Single-cell suspensions of dental pulp and bone marrow were seeded into 6-well plates with alpha modification of Eagle's medium supplemented with 20% FCS 100 mM L-ascorbic acid 2-phosphate 2 mML-glutamine 100 units/ml penicillin 100 mg/ml streptomycin , and then incubated at 37°C in 5% CO2. Thus isolated cell lines were used for studying the proliferation rate of subconfluent cultures (first

subculture) of DPSCs and BMSCs were accessed and also utilised for immuno histochemistry.

Gronthos S, Brahim J, Li W, et al in *2002* [13] followed a similar isolation method for DPSCs as an attempt to study their stem cell properties.

Later **Shi S and Gronthos S in 2003**[2] also followed a similar method of isolation of dental pulp stem cells as an attempt Sto identify the stem cell niche of DPSCs. They used magnetic and fluorescence activated cell sorting for STRO1 positive cells.

In **2006 Cazaux SL, Bluteau G, Magne D et al** [14]**,** in order to determine the optimal culture conditions of DPSCs used both minimal essential medium as well as Roswell Park Memorial Institute medium 1640 (RPMI 1640). They concluded that culture medium plays a role in human pulp cell behaviour. Minimal Essential Medium (MEM) seems to be a more potent culture medium in terms of cellular proliferation, percentage of smooth muscle actin positive cells and odontoblastic differentiation than that of RPMI 1640. The main difference between these media is their calcium/phosphate contents.

Yu J, Wang Y, Deng Z et al 2007 [15] cultured the pulp cells isolated from the incisors of four-week-old Sprague–Dawley rats in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS), 0.292 mg/ml glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, 2.5 μg/ml ascorbic acid and 25 mg/l bovine pituitary extract . This study was to compare the

dental and non dental based stem cell based tooth engineering, in which DPSCs appear to be more competent than BMSSCs in bioengineered teeth.

Morito A, Kida Y, Suzuki K et al 2009 [16] followed a similar method of isolation as previously described by **Gronthos S, Mankani M, Brahim J et al 2000** [9] to study the consequence of basic fibroblast growth factor (bFGF) on the evolution of the stem cell characteristics of human dental pulp stem cells. The study employed microscope based laser scanning cytometer (LSC) (Olympus, Tokyo). Like flow cytometry, LSC facilitates the quick and extremely precise measurement of large number of cells. The differential potential of the cultivated stem cells was identified using mesenchymal function identification kit (R & D systems, Minneapolis, USA). Akiyuki Morito et al found that ratio of cells and cell numbers in the S phase was significantly higher in those cells incubated with bFGF than those incubated without bFGF.

Suchanek J, Soukup T, Visek B et al 2009 [17] in order to determine the best composition of cultivation media for Dental Pulp Stem cells (DPSCs), compared the phenotypes and basic biological properties of DPSCs cultivated in different cultivation medium. Considering the potential clinical uses of DPSCs Fetal Calf Serum (FCS) are to be avoided. The study involved the analysis of various composition of FCS and Insulin, Transferin and Sodium selenite (ITS) supplement. DPSCs were cultivated in 5 % CO2 atmosphere under 37 °C in three different media.

The first was composed of alpha modification of minimal essential medium (α-MEM) , 2 % FCS , 10 ng/ ml EGF , 10ng/ml PDGF, L-ascorbic acid , 2 % glutamine, penicillin/streptomycin, gentamycin and dexamethasone .

The second was composed of α-MEM, 2 % FCS, 10ng/ ml EGF, 10 ng/ml PDGF, L-ascorbic acid, glutamine, penicillin/streptomycin, gentamycin, dexamethasone and supplemented with 10 μl/ml ITS .

The third medium was composed of α-MEM, 10 % FCS, 1 % L-ascorbic acid, glutamine, penicillin/streptomycin and gentamycin.

Cell viability and other biological characteristics were examined episodically by means of a Vi-Cell analyzer and Z2-Counter. DNA study and phenotyping were done using flow cytometry.

The researchers concluded that culture medium did not influence the cell viability and degeneration. However medium containing 2 % FCS supplemented with ITS provided better cultivation conditions for DPSCs (shorter doubling time and more stable proliferation activity were measured) than other tested media. Decrease in concentration of FCS and adding ITS into media had no negative effects on basic biological characteristics (viability, cell diameter). DPSCs cultivated in 2 % FCS with ITS showed lower positivity mesenchymal stem cell markers and HLA I compared to DPSCs cultivated in 2 % FCS or 10 % FCS media.

Manikandhan R, Muthu M S, Sunil PM et al 2010 [18] **Eslaminejad MB, Nazarian H, Shariati M et al 2010**[19] sucessfully isolated and characterized dental pulp stem cells by using 15% FBS in Dulbecco's Modifications of Eagle's Media (DMEM) except that former used type I & II collagenase for the enzymatic action of the extirpated pulp.

STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED)

Miura M, Gronthos S, Zhao M et al in 2003[1] successfully isolated and characterized the stem cells from deciduous teeth. Normal exfoliated human deciduous incisors were collected from 7- to 8-year-old children under approved guidelines set by the National Institutes of Health Office of Human Subjects Research. The pulp was separated from a remnant crown and then digested in a solution of 3mg/ml collagenase type I and 4mg/ml dispase for 1 h at 37°C. Further single cell suspension and cultivation was done according to the method followed by **Gronthos, Mankani M, Brahim J, et al 2000** [9]

Gronthos S, Mankani M, Brahim J et al 2007 [20] isolated the Stem cells from Human Exfoliated Deciduous teeth (SHED) and emphasized the perivascular origin of these stem cells using the expression of STRO1 and CD 146. Deciduous teeth with half extracted roots were obtained from dental centre and were immediately washed in Phosphate Buffered Saline. Cold trypsin was used to incubate the cells for enzymatic action. Isolated cells were passed through a 70µm filter and were cultured in a Dulbecco's modification of eagle's medium (DMEM),Fetal Calf Serum (FCS) 15%, 100U/ml, penicillin 100µg/ml streptomycin. **Peneva M, Mitev V, Ishketiev N 2008** [21] successfully isolated

Stem cells from Human Exfoliated Deciduous teeth (SHED). The method of original cultivation through enzymatic separation by means of trypsin and collagenase in a medium of Dulbecco's Modified Eagle Medium/ Ham's F12 (DMEM F12) was applied. Immediately after the extraction, the deciduous tooth were treated with collagenase V or trypsin and was left in a medium of DMEM F12.

Alipour R, Sadeghi F, Beni BH et al 2010 [22], in an attempt to compare the characteristics of two mesenchymal stem cell population from two different sources like adipose tissue and deciduous teeth, isolated SHED from deciduous teeth. The extracted pulp tissues from normal exfoliated deciduous teeth of six to nine year old children digested in collagenase type I for 1 hour at 37°C. The harvested cell suspensions were filtered through a 40 μm cell strainer and the single cell suspensions were cultured in Dulbecco's Modification of Eagle's medium supplemented with 10% Fetal Calf Serum at 37°C with 5% CO2.

Kadar K, Kiraly M, Porcsalmy B et al 2009 [23] evaluated the importance of serum in the media and concluded that FBS stimulated cell proliferation compared to serum-free controls.

BIOLOGICAL CHARACTERISTICS

Based on the kinetic studies conducted by **Gregory CA, Singh H, Perry AS et al 2003**[24] on Human Adult Stem Cells from Bone Marrow.

3 phases in MSC growth were observed

(1) An initial lag phase

(2) Rapid expansion

(3) A stationary phase.

Luo J, Chen J, Deng ZL et al 2007[25] reviewed the various studies on Wnt signalling in human disease and found that de-regulation of Wnt (drosophila segment polarity gene Wingless and the mouse proto-oncogene Int 1) pathways alters the homeostatic balance of self renewal in adult tissues. Of the three pathways of Wnt signalling canonical pathway seem to play a role in Mesnchymal Stem Cell (MSC) differentiation. Wnt plays an induction role in osteogenic differentiation, chondrogenesis, adipogenesis and myogenesis. The transdifferentiation of myoblasts to adipocytes is also affected by the disruption of Wnt signalling.

Gregory CA, Singh H, Perry AS et al 2003 studies indicated that dickkopf-1 (DKK-1) inhibits the positive signalling through the canonical Wnt/ β catenin pathway by binding to lipoprotein related proteins . This binding actually disrupts the frizzled receptor complex thus decreasing both nuclear and cytoskeletal β catenin that plays a role in transcription and formation of adherent junctions respectively. Thus increased DKK – 1 reduced the levels of β catenin resulting in decreased cell proliferation and differentiation. [24]

PHENOTYPIC CHARACTERIZATION

Studies on rat skin and lung fibroblasts revealed the presence of three subpopulations of cells (**Mollenhauer J and Bayreuther K 1986**) [26] **[Annexure VI]**

i. F I – Spindle shaped cells with high proliferation potential
ii. F II – Epitheloid cells with comparatively lower proliferation rate

iii. F III - Large stellate cells that proliferated slower than other types

Studies by **Bayreuther K, Rodemann HP, Hommel R et al 1988** [27] on human skin fibroblasts of cell lines isolated from the lower abdominal region, provided evidence that fibroblasts *in vitro* can spontaneously differentiate into a seven stage terminal cell lineage as follows. **[Annexure –VII]**

Mitotic fibroblast subtypes

i. F1 – small spindle shaped cells

ii. F2 – small epitheloid cells

iii. F3 – larger pleomorphic epitheloid cells

Postmitotic subtypes

i. F4 – large spindle shaped cells

ii. F5- larger epitheloid cells

iii. F6- largest epitheloid cells

iv. F7-degenerating fibroblasts

Suchánek J, Víšek B, Soukup T et al 2010 [28] did comparison of Dental Pulp Stem Cells (DPSCs) and Stem cells from Human Exfoliated Deciduous teeth (SHED). Both SHED and DPSCs had similar average diameter. (SHED 15.0 μm and DPSC 15.2 μm). But the diameter distribution of SHED varied in a wider range (12.24–16.43 μm) compared that of DPSC (14.25–16.13 μm). Also it was analysed that SHED had 45 Population Doubling (PD) counted from the 2nd passage. In primary cultures the amount of cells was very low; therefore the counting didn't begin with the first passage to enhance the yield and number of cells seeded. In contrast DPSC showed a PD of only 10 though it was counted from the first passage as number of cells in the primary cultures on an average

was 50. In comparison to DPSC, SHED had higher average Doubling Time (DT). The average SHED DT was 41.3 hrs (21.3–97.3 hrs) compared to DPSC 24.5 hrs (15.55–35.12 hrs). For the first 24 PD, SHED DT (28.4 hours) was about 33 % higher than DT of DPSC (19.3 hours). After reaching 24 PD DT of SHED DT increased to 54.2 hours, while PD of DPSC increased only to 29.1 hours.

The above study state that dental pulp of exfoliated teeth thereby represents alternative and easily accessible source of tissue-specific stem cells which are histocompatible with patient specific tissues. SHED were stable after cryopreservation and can be therefore be quite useful for stem cell tissue banks. Dental pulp tissue from exfoliated deciduous teeth represents an easily accessible source of tissue; one which is often discarded, but which instead may be useful as a source of stem cells for research and clinical applications.

On the contrary to somatic cells, the stem cells may be propagated over Hayflick's limit. Replicative capacity in somatic cells is restricted due to a steady decline in telomere length with each round of DNA replication that ultimately stops cell division and causes senescence. The stem cells gained an advantage over somatic cells and evolved mechanisms that could counteract telomere loss. They express a specific ribonucleoprotein enzyme, telomerase, which is required for telomere length maintenance [29].

IMMUNO PHENOTYPIC CHARACTERIZATION

As a consequence of the great quantity of antibodies and kits from various molecular laboratories and mercantile suppliers, immunostaining, western blot and Enzyme Linked Immuno Sorbent Assay (ELISA) assays are in the midst of the

most valuable techniques accessible for cell line characterization . Antibody localization is accessed either by fluorescence, in which the antibody is conjugated to a fluorochrome, such as fluorescein or rhodamine, or by accessing precipitated product deposited of a precipitated product from the activity of horseradish peroxidase or alkaline phosphatase conjugated to the antibody [30] .

A systematic review on cell surface characterization of adult mesenchymal stem cells by **Mafi P, Hindocha S, Mafi R et al 2011 [31]** was done. Mafi et al concluded that there are various surface markers for mesenchymal stem cells like CD105, CD90, CD44, CD73, CD29, CD13, CD34, CD146, CD106, CD54 and CD166 had a positive expression wheareas the antibodies such as CD14, CD11b, CD49d, CD34, CD106, CD10 and CD31 have been reported to have a negative expression. It was also observed that in all the studies considered for this review markers like CD10, CD34, CD45 and CD106 did not have a uniform expression in the cell types and it varied among the different studies. The variability of the expression of the markers can be attributed to the heterogenicity of the cell types, or to the different cell passages that was used to access the expression of markers.

STRO1**[32]** is one of the early cell surface markers for mesenchymal stem cells that can be used to evaluate the undifferentiated status of DPSCs and SHED. Monoclonal antibody for STRO1 was first described as a potential reagent that reacted with a cell surface molecule highly expressed on human bone marrow Colony Forming Unit of Fibroblast (CFU-F) Dental Pulp Stem Cells (DPSCs) and Stem cells from Human Exfoliated Deciduous teeth (SHED) have been found to contain a STRO1-positive fraction at around 10–20% **Gronthos S, Mankani M, Brahim J et al 2000 [9]**; **Miura M, Gronthos S, Zhao M et al 2003[1]**. CD146

(MUC18), known as a possible marker for Bone Marrow derived Mesenchymal Stem cells (BM-MSCs), is also expressed in DPSCs and SHED **Gronthos S, Mankani M, Brahim J et al 2000[9] Miura M, Gronthos S, Zhao M et al 2003[1] Shi and Gronthos 2003[2]**. These markers can be used in immunocytochemistry or Fluorescent Activated Cell Sorting analysis (FACS).

As per the review by **Kolf CM, Cho E and Tuan RS 2007[6]** Stro-1 is by far the best-known Mesenchymal Stem Cell (MSC) marker. The cell population negative for Stro-1 is not capable of forming colonies that is, it does not contain Colony Forming Unit (CFU-Fs) Stro-1- positive cells can become Hematopoietic Stem Cells (HSC) -supporting fibroblasts, smooth muscle cells, adipocytes, osteoblasts, and chondrocytes. However, Stro-1 is unlikely to be a general MSC marker, for three reasons: first, there is no known mouse counterpart of Stro-1; second, Stro-1 expression is not exclusive to MSC as it is also expressed by the whole cell population of human breast milk[33] human skeletal cells , tumor initiating cells of osteosarcoma [34] , bone marrow aspirates and third, its expression in Mesenchymal Stem Cells (MSCs) is gradually lost during culture expansion , limiting the use of Stro-1 tagging to the isolation of MSCs and/or their identification during early passages. As the exact function of the Stro-1 antigen is unknown, it is unclear whether loss of Stro-1 expression alone has functional consequences for MSC stemness. Application of Stro-1 as an MSC marker is therefore best done in conjunction with other markers.

CD106, or VCAM-1 (vascular cell adhesion molecule-1), is expressed on blood vessel endothelial and adjacent cells, consistent with a perivascular location of Mesenchymal Stem Cells (MSC). It is likely to be functional in MSCs because

it is involved in cell adhesion, chemo taxis, and signal transduction, and has been implicated in rheumatoid arthritis . CD106 singles out 1.4% of Stro1-positive cells, increasing the CFU-F frequency to 1 in 3, which are all high Stro1-expressing cells and are the only Stro1-positive cells that form colonies and show stem cell characteristics such as multipotentiality, expression of telomerase, and high proliferation *in vitro*. Taken together, these data suggest that Stro1 and CD106 combine to make a good human MSC marker.

Mesenchymal stem cells represent only about 0.001%-0.01% of the total cells. However, the therapeutic application of MSCs often requires a large number of cells, which requires ex vivo expansion post-harvest [35].

In order to facilitate a more unified approach to studying MSC biology, **The International Society of Cryotherapy** has devised three criteria needed to identify MSCs [35]:

1) Plastic adherence of the isolated cells in culture
2) expression of cluster of differentiation (CD) markers such as CD105, CD73, and CD90 in > 95% of the culture with absent expression of markers including CD34, CD45, CD14 or CD11B, CD79A or CD19 and human leukocyte antigen-DR (HLA-DR) in > 95% of the culture and
3) Capacity to differentiate into osteocytes, adipocytes and chondrocytes.

Immunohistochemical studies were performed to characterize the progeny of the Dental Pulp Stem Cell (DPSC) and Bone marrow derived Stem Cell (BMSC) clonogenic populations (**Gronthos S, Mankani M, Brahim J et al 2000 [9])**, by using a large panel of antibodies specific to known antigens associated with

different phenotypes. Primary cultures of DPSC and BMSC failed to react with the hematopoietic markers CD14 (monocyte macrophage), CD45 (common leukocyte antigen), CD34 (hematopoietic stem progenitor cells endothelium), and other markers such as MyoD (smooth muscle), neurofilament (nerve), collagen type II (cartilage), and Peroxisomal Proliferator Activated Receptor Gamma 2 (PPARγ2) (fat). In general, DPSCs and BMSCs exhibited a similar expression pattern for a variety of markers associated with endothelium vascular cell adhesion molecule 1 and MUC-18 (CD146), smooth muscle (α-smooth muscle actin), bone (alkaline phosphatase, type I collagen, osteonectin, osteopontin, and osteocalcin), and fibroblasts (type III collagen and fibroblast growth factor 2). The bone matrix protein, bone sialoprotein, was absent in DPSC cultures, but present at low levels in BMSC cultures. Many of the markers were not uniformly expressed, but found in subsets of cells, indicating that the DSPC population is heterogeneous, as has been shown for the BMSC population. The DPSC cultures were also found to be negative for the odontoblast-specific marker Dentin SialoPhospho protein (DSPP), by Northern blot analysis, which is suggestive of an undifferentiated phenotype.

Kerkis I, Kerkis A, Dozortsev D et al 2006 [36] successfully isolated dental pulp stem cells that expressed the various embryonic stem cell markers Oct-4, Nanog, Stage Specific Embryonic Antigen (SSEA) -3, SSEA-4, TRA-1-60 and TRA-1-81 (both TRA 1-60 and TRA -1 81 are expressed on human tetra carcinoma stem cells) as well as several other mesenchymal stem cell markers during at least 25 passages while maintaining the normal karyotype and the rate of expansion characteristic of stem cells.

Bowen A, English A, Jones E et al 2006 [37] based on their observation of human DPSCs expressing surface molecules similar to mesenchymal stem cells concluded that pluripotent stem cells are present within the stem cells .

Wei X, Ling J, Wu L et al 2007 [38] investigated mineralization capacity of human dental pulp cells and identify the potential markers for odontoblastic differentiation. The isolated DPCs expressed mesenchymal stem-cell markers as shown by flow cytometry. Quantitative RT-PCR revealed that osteocalcin, dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE) expression also increased time dependently in the induction cultures. Xi Wei et al conclude that MEPE along with DSPP may be potential odontogenetic differentiation markers.

According to **Kadar K, Kiraly M, Porcsalmy B et al 2009** [23] a fraction of the cells in DPSC expressed the cell surface molecule STRO1, a mesenchymal stem cell marker. STRO1 immunoreactivity gradually decreased with increasing passage numbers but 6-8% of the cells were still STRO1 positive even at higher passage numbers.

Karbanová J, Soukup T, Suchánek J et al 2010 [39] successfully isolated and expanded dental pulp stem cells (DPSC) from third molars and analyzed the expression of various markers by flow cytometry, immunocytochemistry, immune blotting. Flow cytometry showed DPSCs were positive for mesenchymal markers but not for hematopoietic markers. Immunocytochemical revealed the positive expression of numerous stem cell markers, including nanog, Sox2, nestin, Musashi-1 and nucleostemin and negative expression of differentiated neural,

vascular, and hepatic cells. Immunoblotting analyses also revealed similar results. These cells showed slight expression of smooth muscle actin and variable expression of CD 146.

Alipour R, Sadeghi F, Beni BH et al 2010[22], phenotypically characterized and compared Stem cells from dental pulp of exfoliated deciduous teeth (SHED) and adipose tissue. Staining by different fluorescent labelled monoclonal antibodies against surface markers was analyzed using flow cytometry. Although the range of the considered cell surface markers is substantially wide, nevertheless a general overlook of them, infers that Mesenchymal Stem cell (MSC) derived from multi tissues and organs have some phenotypes in common. Among the markers that have been more consistently reported, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have established certain panel of cell-surface markers including the following: about 95% of the MSC population must express CD73, CD90 and CD105, and no more than 2% of the cells express CD34, CD45, CD11b or CD14, CD19 or CD79a and HLA-DR. Both cell types expressed CD90 and CD44 (cell adhesion receptor) at high levels. They did not express CD45 (common leukocyte antigen), CD14 (monocyte and granulocyte marker), CD11b, CD19, CD34 (hematopoietic cell lineage marker) and CD3 (lymphocyte antigen). In addition, SHED expressed CD105 (Endoglin, SH2). CD146 or MCAM (Melanocyte Adhesion Molecule) or MUC18, is a highly glycosylated transmembrane glycoprotein part of the immunoglobulin (Ig) superfamily, initially recognized in melanoma cells.

DIFFERENTIATION POTENTIAL

Yu J, He H, Tang C et al 2010 [5] evaluated the differentiation potential of DPSCs at different passages. For this they compared STRO1 positive cells from 1st and 9th passage. As compared with polymorphic DPSCs at the 1st passage (DPSC-P1), some DPSCs at the 9th passage (DPSC-P9) displayed the enlarged cell bodies and elongated appearance with one or two long cellular processes. Down regulated proliferation potential was concluded due to the prolonged population doubling time in the 9th passage. The phenotypic changes were evaluated based on RT –PCR and western blot. The gene expression of alkaline phosphatase (*ALP*, mature osteo-/odontoblast marker), osteopontin (*OPN*, mature osteoblast marker), osterix (*OSX*, mature osteoblast marker), bone sialoprotein (*BSP*, mature osteoblast marker), dentin sialophosphoprotein (*DSPP*, odontoblast marker), and type II collagen (*COL* II, chondrocyte marker) was significantly up regulated in the 9th passage, in comparison with the 1st passage.

Miura M, Gronthos S, Zhao M et al in 2003[1] as an attempt to investigate the potentiality of Stem cells from Human Exfoliated Deciduous teeth (SHED) to differentiate into mineralized tissue, established secondary SHED cultures were supplemented with L-ascorbate-2-phosphate, dexamethasone and inorganic phosphate. Accumulation of calcium nodules was confirmed by alizarin red positivity. The up regulation of various bone markers Core Binding Factor subunit Alpha -1 (CBFA1), Alkaline Phoshatase , Mareix Extracellular Phospho Glyco protein (MEPE), and bone sialoprotein were up-regulated after induction was confirmed in western blot . Also, Dentin Sialo Phospho Protein (DSPP) was induced by the mineralizing induction . Furthermore, Bone Morphogentic Protein

(BMP-4) treatment was capable of inducing an up-regulated expression of CBFA1, Osterix, and Osteocalcin by semiquantitative RT-PCR . These data indicated that SHED possessed the ability to differentiate into functional odontoblast like cells *in vitro*. The authors also found that cultured SHED expressed a variety of neural cell markers including nestin III-tubulin, GAD, NeuN, GFAP, NFM, and CNPase as measured by immunocytochemical staining and Western blot analysis.

Although DPSCs remained stable in their viability, phenotype, and genotype over cultivation, progressive telomere shortening detected by **Mokry J, Soukup T, Micuda S et al 2010** [29] indicated that adult tissue-specific stem cells might be at risk of detrimental effects caused by excessive ex vivo expansion. The study further demonstrated that *in vitro* conditions DPSC lines are capable of long-term cultivation without changing their viability, phenotype, and genotype. DPSCs expressed many stem cells markers in distinct pattern from other adult stem cells. DPSCs did not express haematopoietic markers CD34 and CD45, but expressed a high level of mesenchymal markers such as vimentin, STRO1, CD29, CD44, CD73, CD90 and CD166 . Expression of pluripotent embryonic stem cell marker, Sox-2 confirms the primitive nature of DPSCs. Neural stem cell markers such as nestin and nucleostemin observed in DPSCs may reflect the neural crest origin of the dental pulp. DPSCs have been also described to express astroglial marker GFAP. Apart from the various expressions of stem cell markers by DPSCs and proof of their multipotency, their extensive proliferative capacity in long-term culture was also documented

IN VIVO EXPANSION

In order to study the effectiveness of these stem cells especially the mesenchymal stem cells for therapeutic applications it is essential to study their potential *in vivo* especially study models. It generally involves the transplantation of *in vitro* expanded cells into suitable scaffolds especially immune compromised mice. After few weeks the transplants are recovered and stained to study the various structures.

Gronthos S, Mankani M, Brahim J et al in 2009[9] transplanted the mixture of Dental Pulp Stem Cells (DPSC) and hydroxy appatite and tri calcium Phosphate (HA / TCP) into the dorsal surface of 10 week old immunocompromised mice. This resulted in a dentin like structure on the surface of HA/TCP. This dentin like Structure when observed under polarizing microscope revealed collagenous matrix arranged perpendicular to odontoblastic like layer and it was composed of Type I collagen, that was proved immunologically.

Miura M, Gronthos S, Zhao M et al in 2003[1] transplanted the isolated SHED into immune compromised mice. This yielded human-specific alu-positive odontoblasts directly associated with a dentin-like structure. Importantly, the regenerated dentin was immune reactive to dentin-specific DSPP antibody. Neural developmental potential was studied further *in vivo* by injecting SHED into the dentate gyrus of the hippocampus of immunocompromised mice. Histological examination showed that SHED survived for 10 days inside the mouse brain microenvironment as noted by human-specific antimitochondrial antibody staining and continued to express neural markers such as Neural Filament (NFM).

Koyama N, Okubo Y, Nakao K et al 2009 [40] evaluated the pluripotency of postnatal stem cells like Dental Pulp Stem Cells (DPSC) and Stem cells From Human Exfoliated Deciduous teeth (SHED). The isolated cells were further incubated and tested for osteogenic potential by treatment with bone morphogenetic protein (BMP). BMP treatment groups produced alkaline phosphatase in the cells and also secreted osteocalcin in the culture medium. The cells were also tested for adipogenic differentiation, where it was found that there was potential for SHED and DPSC to express 2 adipocyte-specific transcripts , proliferator-activated receptor γ2 (PPARγ2) and lipoprotein lipase (LPL). Upregulated expression of Osteocalcin or Sec determining region Y – Box 9 **(**Sox9) Col 2, and Col X were observed by reverse transcriptase polymerase chain reaction. This study demonstrated that pluripotential cells isolated from the pulp of human teeth (DPSC, SHED) expanded *in vitro* and differentiated into osteoblasts, chondrocytes, and adipocytes.

Yu J, He H, Tang C et al 2010 [5] transplanted rat DPSC pellets at the 1st and 9th passages in to the renal capsule for adult rats . *In vivo* transplantation results showed that all 1st passage cell pellets gave birth to woven bone tissues. Among those samples there was simultaneous production of dentin structures with typical odontoblasts lining inner side of pre dentin, cartilage structure with chondrocytes, distinct osteoblasts and osteocytes seen around or inside bone structures. In case of the 9th passage there was development of bone tissues with thicker matrix, less lacunae and less osteocytes with no osteoblasts.

Nourbakhsh N, Soleimani M, Taghipour Z et al 2011 [41] evaluated to prove the *in vitro* differentiation of SHED into neural cell lineages. They isolated the SHED using enzyme disaggregation technique from the pulp of deciduous teeth. The cells thus isolated had typical fibroblastoid morphology and expressed antigens characteristic of MSCs, STRO1, CD146, CD45, CD90, CD106 and CD166, but not the hematopoietic and endothelial markers, CD34 and CD31, as assessed by FACS analysis. A simple short time growth factor-mediated induction was used. Immuno fluorescence staining and flow cytometric analysis revealed that SHED rapidly expressed nestin and b-III tubulin, and later expressed intermediate neural markers. In addition, the intensity and percentages of nestin and b-III tubulin and mature neural markers (PSA-NCAM, NeuN, Tau, TH, or GFAP) increased significantly following treatment. Moreover, RT-PCR and Western blot analyses showed that the neural markers were strongly up-regulated after induction. Thus it was finally concluded that SHED was capable of differentiating into neural cells. SHED cells might be considered as new candidates for the autologous transplantation of a wide variety of neurological diseases and neuro traumatic injuries.

APPLICATION:

Stem cells have had an enormous impact on biology and medicine. Embryonic Stem cells (ES) have been used to study specific signals and differentiation steps required for the development of many tissues.

1. ES cells made possible the production of knockout mice. To produce these mice, a specific gene is inactivated or deleted from cultured ES cells. These cells are injected into blastocysts, which are then implanted into the uterus of a surrogate mother. The genetically modified implanted

blastocysts develop into full embryos, as long as the gene defect does not cause embryonic lethality. Knockout mice have become widely used models for the experimental study of human disease and provide essential information about gene function *in vivo*.

2. Testing the efficacy of drugs
3. Cell based therapies in acute and chronic degenerative diseases such as
    - ✓ Parkinson's and Alzheimer's disease.
    - ✓ Spinal cord injury
    - ✓ Burns
    - ✓ Heart diseases
    - ✓ Diabetes
    - ✓ Osteo/Rhematoid arthritis
    - ✓ Liver diseases
4. Disease modelling

Results

This study was done to analyze the growth, phenotypic and surface characteristics of the morphologically characterized stem cells from the pulp of permanent and deciduous teeth. We isolated dental pulp cells from 12 samples of permanent teeth and 9 samples of deciduous teeth. **[Annexure –I, II]** The tooth was collected in the transport medium after surgery or extraction. The transport media was α modification of minimal essential media without serum with twice the concentration of antibiotics added to prepare working media i.e., Penicillin-100 IU, Streptomycin-100µg/ml. Of all these samples considered, we were able to culture successfully 5 dental pulp stem cell populations and 6 stem cells from exfoliated deciduous tooth cell populations. Based on the yield obtained from each culture we concluded that collection of the tooth immediately in the transport media was more important than processing the tooth collected in the transport media. Duration for enzymatic disaggregation for the pulp tissue from permanent teeth and deciduous teeth were 17 hours and 5 to 6 hours respectively. On observing the disaggregated pulp tissue 48 hours after plating , cluster of cells are seen arising from each foci of cells **(figure 1)** or single cells **(figure 2)** scattered in the plate. Size of the colonies were determined based on the number of cells (up to 10 cells were considered to be small colonies and more than 10 colonies were considered to be large colonies). The plates were henceforth observed daily until confluency **(figure 3)** was reached. Meanwhile media change was done every third day. The primary culture reached 70% to 80% confluency in about 21 to 35 days. The first subculture derived cells took about 7 to 10 days to reach confluency. The second subculture derived cells took about 4 to 8 days. The third subculture derived cells took about 5 to 8 days to reach confluency. Cells from the 3^{rd} to 4^{th} passage were used to study the growth characteristics and subpopulation

analysis. The fourth subculture derived cells took about 4 to 15 days. The plates considered for subpopulation analysis took about 15 days to reach confluency as only minimal cells, 5 x 10^3 cells were plated. Immunocytochemistry analysis was done for STRO1 and CD106 was done for all the samples considered for Dental Pulp Stem Cells and Stem cells from Human Exfoliated Deciduous teeth.

TROUBLE SHOOTING OF PERMANENT TOOTH SAMPLES CONSIDERED FOR DENTAL PULP STEM CELLS.

Permanent Sample 1

Impacted permanent molar of a 30 year old female was received in the transport media (α modification of minimal essential media with antibiotics but without serum) 15 minutes after extraction. The pulp obtained was subjected to disaggregation in the enzymatic solution containing 2mg of crude collagenase and 1mg of dispase in 1 ml of working media (α modification of minimal essential media with serum and antibiotics). After 17 hours of enzymatic action the tissue was plated in a tissue culture plate. When observed after 48 hours the plate showed granularity. When this granularity was observed under microscope numerous bud like structure were seen **(figure 4)** suggestive of fungal contamination. The plate was immediately discarded. The probable cause of contamination was attributed to delayed collection of tooth after extraction, in the transport media. As a result of which the tooth was exposed to the environment.

Permanent sample 4

Impacted permanent molar of 22 years old female was obtained in a transport media immediately after surgery. The sample was immediately processed to extirpate the pulp. The pulp was subjected to disaggregation in the

enzymatic solution containing 2mg of type I collagenase and 1mg of dispase in 1ml of working media. After 17 hours of enzymatic action the tissue was plated in a tissue culture plate. Few small colonies were observed in the first week **(figure 5)**. In the consecutive days the there was an increase in size and number of colonies. As the cells were reaching confluence in about 25 days **(figure 6)** few hyphae like structures were observed **(figure 7).** The plate was immediately discarded.

Permanent sample 5

Impacted permanent molar of a 35year old female was transported to the laboratory in the transport media, immediately after surgery. The extirpated pulp was subjected to disaggregation in the enzymatic solution containing 2 mg of crude collagenase and 1mg of dispase in 1 ml of working media. After 17 hours of enzymatic action the tissue was plated in a tissue culture plate. Very few colonies were found in the first week. The size of these colonies was extremely small except for 2 or 3 colonies. More number of single cells was observed. Although spindle shaped cells were predominantly epitheloid and stellate shaped cells were also equally present. By the end of second week, hyphae like structures were seen suggestive of fungal contamination. The plate was immediately discarded. At the same time cotton like fluffy substances were found in the water tray of the CO_2 incubator. The material was taken in the slide and stained for Haematoxylin and eosin. The substance was identified to be Aspergillum. As precaution to prevent fungal contamination in future the water in the incubator was immediately discarded, the tray was washed completely with soap, after drying the tray was wiped with alcohol and kept in the UV for about 20 minutes and double

autoclaved deionized water was then added to it and kept in the same UV for about 40 minutes and finally the tray was taken into the incubator.

Permanent sample 7

Impacted permanent molar from a 28 year old male was transported to the laboratory for processing immediately after surgery. The pulp obtained was subjected to disaggregation in a enzymatic solution containing 2mg of TYPE I collagenase and 1mg of dispase 1 ml of working media. Very few colonies were observed in the first week. But soon filamentous structures were seen floating in the media which was characteristically appeared like fungal hyphae under microscope. The plate was discarded.

Permanent sample 8

Impacted permanent molar from a 22 year old female was carried immediately to the culture lab for immediate processing in the transport media. The enzyme solution containing 2mg of Type I collagenase and 1mg of dispase 1 ml of working media was used for the disaggregation of the extirpated pulp. After the primary culture there were no colonies seen in the first week except for few cells. In second week few colonies were found. Few cells and colonies were seen later. After few weeks hyphae like structures were seen under the microscope **(figure8).** The plate was discarded. The water in the incubator was replaced by double autoclaved deionized water. The incubator was completely wiped with alcohol.

Permanent sample 10

Impacted permanent molar from a 27 years female, was transported immediately after surgery to the cell culture laboratory in the transport media. The enzyme solution containing 2mg of Type I collagenase and 1mg of dispase in 1ml working media was used for the disaggregation of pulp tissue obtained from the tooth. On observation 48 hours after plating the primary culture showed no colonies. The plate showed very few cells and debris. Finally 5^{th} day it had filamentous structures floating which appeared like branched hyphae with septae under microscope. Such structures when taken in a slide for staining were Periodic acid Schiff positive.

Permanent sample 11

Impacted permanent molar from a 28 years old male was transported immediately after surgery to the cell culture laboratory in the transport media. The enzyme solution of 2mg of Type I collagenase and 1mg of dispase in 1ml of working media was used for the disaggregation of pulp tissue obtained from the tooth. It had few colonies on observation on the 2^{nd}, $3^{rd\ and}$ 4^{th} day. But few days later the thick yellowish white structure were seen floating in the media attached to one corner of the plate was seen. On observation under microscope highly branched filamentous structure suggestive of fungal contamination was seen. The plate was immediately discarded.

TROUBLE SHOOTING IN THE DECIDUOUS TOOTH SAMPLES CONSIDERED FOR STEM CELLS FROM HUMAN EXFOLIATING DECIDUOUS TEETH.

Deciduous sample 2(b)

An exfoliated molar from a 10 years old male with caries was obtained in the transport media immediately after removal. On extirpating, the pulp was found that unlike the radicular pulp the coronal pulp was slimier and yellowish henceforth it was considered to be another sample. The pulp thus considered was subjected to 5 hours of enzyme disaggregation in the enzymatic solution containing 2mg of Type I collagenase and 1mg of dispase in 1ml of working media. On observing 48 hours after plating the plate under microscope revealed numerous filamentous structures suggestive of fungi was floating. These filamentous structures which when taken on a slide, stained positive for periodic acid Schiff.

Deciduous sample 7

An exfoliating deciduous mandibular incisor from a 8 year old male with root minimally resorbed was transported immediately to the cell culture laboratory for processing in transport media. The pulp obtained was subjected to 6 hours of enzyme disaggregation in the enzymatic solution containing 2mg of Type I collagenase and 1mg of dispase in 1ml of media. 48 hours after plating of primary culture, 2 or 3 small colonies were observed. After 7 days the plate had white particles floating. These particles appeared like filamentous fungal hyphae under microscope, suggestive of fungal contamination. The plate was discarded immediately.

Deciduous sample 8

An exfoliating deciduous mandibular incisor from 11 years old male was obtained in the transport media immediately after removal from the mouth. The tooth had 75 % of the root resorbed. The minimal amount of pulp obtained was subjected to 5 hours of enzyme disaggregation in the enzymatic solution containing 2mg of Type I collagenase and 1mg of dispase in 1ml of working media. 48 hours after plating of the primary culture, no colonies were observed except for a very few cells. By the end of first week fungus like structures were seen floating in the medium and the plate was discarded immediately.

Deciduous sample 9

An exfoliated deciduous canine from a 11 years old male was collected in the transport media immediately after removal from the mouth. The tooth had 90% of root resorbed. The very minimal pulp obtained from the tooth was subjected to disaggregation in an enzymatic solution containing 2mg of Type I collagenase and 1mg of dispase in 1ml of working media for about 5 hours. 48 hours after plating of primary culture the plate appeared granular with naked eye. Under microscope the plate was observed to be full of budding hyphae like structures, **(figure 9)** suggestive of fungal hyphae. The plate was discarded immediately.

GROWTH CHARACTERISTICS ANALYSIS

Dental Pulp Stem Cells (Permanent Tooth Sample)

Permanent sample 2 **(GRAPH 1 & TABLE 1):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. Though there was an increase in number of cells 24 hours after plating there was decrease in number of cells on the third day. Thereafter the number of cells increased exponentially and reached the plateau phase on day 7. Seeding efficiency was calculated to be 112 % and population doubling time as calculated from the slope of the curve was 43.92 hours. .

Permanent sample 3 **(GRAPH 2 & TABLE 2):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. There was an increase in the number of cells from day 1. The seeding efficiency was calculated to be 101.83% and population doubling time as calculated from the slope of the curve was 111.99 hours.

Permanent sample 6 **(GRAPH 3 & TABLE 3):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. There was a decrease in number of cells within 24 hours of attachment of cells. Thereafter there was an increase of number of cells until the day 6. The seeding efficiency was calculated to be 57.41% and the population doubling time as calculated from the slope of the curve was 144.62 hours.

Permanent sample 9 (**GRAPH 4 & TABLE 4):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. There was a decrease in the number of cells within 24 hours of

attachment , thereafter there was a gradual increase in the number of cells. The plateau phase was reached on day 7. Population doubling time calculated from the slope of the curve was 111.32 hours and the seeding efficiency was also calculated to be 37%.

Permanent sample 12 **(GRAPH 5 & GRAPH 5)**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. There was a decrease in number of cells for the first two days later there was a gradual increase in the number of cells until the 7^{th} day. The population doubling time calculated from the slope of the curve was 32.43 hours and the seeding efficiency was calculated to be 63.89%.

Stem Cells From Human Exfoliated Deciduous Tooth (Deciduous Tooth Sample)

Deciduous sample 1 **(GRAPH 6 & TABLE 6):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. There was a gradual increase in the number of cells from day 1 to day 7. The population doubling time calculated from the slope of the curve was 246.79 hours and seeding efficiency was calculated to be 119.42%.

Deciduous sample 2 **(GRAPH 7 & TABLE 7):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. The was decrease in number of cells within 24 hours of attachment and thereafter there was a gradual increase in the number of cells and there was a rapid increase in the number of cells after 5^{th} day with a fall in the rise after 7^{th}

day. The population doubling time calculated from the slope of the curve was 13.51 hours and the seeding efficiency was calculated to be 42.59%.

Deciduous sample 3 **(GRAPH 8 & TABLE 8):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. There was a decrease in number of cells within 24 hours of cell attachment. Thereafter there was a gradual increase in the number of cells daily after 1^{st} day. The population doubling time calculated from the slope of the curve was 68.18 hours and the seeding efficiency was calculated to be 83.33%.

Deciduous sample 6 **(GRAPH 9 & TABLE 9):**

The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. There was a decrease in the number of cells within 24 hours of attachment. There was exponential increase in the number of cells until the plateau phase that was reached on day 7.

The population doubling time calculated from the slope of the curve was 73.87hour. The seeding efficiency was calculated to be 43.5%.

SUB POPULATION ANALYSIS

f1 – f3 "MOLLENHAUER et al 1986" [26]

Dental Pulp Stem Cells (Permanent Tooth Sample)

Permanent sample 2 **(GRAPH 10 & TABLE 10, 11)**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was an increase in the proportion of f1 cells

during the 8 days observation period which was statistically significant ($p < 0.05$). There was a decrease in proportion of f2 cells during the same 8 days observation period which was also statistically significant ($p<0.05$). The decrease in the proportion of f3 cells during the 8 days observation period did not have any statistical significance ($p > 0.05$).

Permanent sample 3 **(GRAPH 11 & TABLE 12, 13):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was no significant change in the proportion of f1 cells during a 8 days observation period ($p>0.05$). The increase in the proportion of f2 cells was not statistically significant ($p > 0.05$). The decrease in the proportion of f3 cells was also statistically insignificant ($p > 0.05$).

Permanent sample 6 **(GRAPH 12 & TABLE 14, 15):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was a decrease in f1 during the 8 days observation period with no statistical significance ($p > 0.05$). There was no significant change in the f2 ($p > 0.05$). The increase in f3 during the same 8 days observation period was also statistically insignificant ($p > 0.05$)

Permanent sample 9 **(GRAPH 13 & TABLE 16, 17):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 consecutive days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was a decrease in the proportion of f1 during the 8 days observation period ($p > 0.05$). There was no significant change in the proportion of f2 during 8 days observation period ($p > 0.05$). There was an increase in the proportion of f3 ($p > 0.05$).

Permanent sample 12 **(GRAPH 14 & TABLE 18, 19):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was decrease in proportion of f1 and f2 ($p > 0.05$) during the 8 days observation period. The increase in proportion of f3 was also statistically insignificant ($p > 0.05$).

Stem Cells From Human Exfoliated Deciduous Tooth (Deciduous Tooth Sample)

Deciduous sample 1 **(GRAPH 15 & TABLE 20, 21):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was significantly no change in the proportion of f1 ($p > 0.05$) during the 8 days observation period. There was increase in the

proportion of the f2 but there was no statistical significance ($p > 0.05$). There was a decrease in f3 that was also statistically insignificant ($p > 0.05$).

Deciduous sample 2 **(GRAPH 16 & TABLE 22, 23):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was a statistically significant increase in the proportion of f1 ($p < 0.05$). The increase in the proportion of f2 cells during the 8 days observation period and the decrease in the proportion of f3 cells during the same observation period were statistically insignificant.

Deciduous sample 3 **(GRAPH 17 & TABLE 24, 25):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was an increase in the proportion of f1 during the 8 days observation period, there was a decrease in the proportion of f2 during the same observation period. The proportion of f3 showed no significant change. But all these observations were statistically insignificant ($p > 0.05$).

Deciduous sample 6 **(GRAPH 18 & TABLE 26, 27):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Mollenhauer et al 1986. There was a increase in the proportion of both f1 and

f2, but the increase were not statistically significant ($p > 0.05$). The decrease in the proportion of f3 was also statistically insignificant ($p > 0.05$).

Subpopulation analysis

F1 to F7 [KLAUS BAYREUTHER et al 1988][27]

Dental Pulp Stem Cells (Permanent Tooth Sample)

Permanent sample 2 **(GRAPH 19 & TABLE 28):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Klaus Bayreuther et al 1988. There was an increase in f1, f3 and f4. But there was a apparent decrease in f2, f6 and f7. Though f5 increased during the 3rd to 5th day there was no change finally.

Permanent sample 3 **(GRAPH 20 & TABLE 29):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Klaus Bayreuther et al 1988. The proportion of f1 showed an increase during the 4th day but finally there was a decrease. There was increase in f2, f4 f6 and f7. There was a decrease in the proportion of f3 and f5.

Permanent sample 6 **(GRAPH 21 & TABLE 30):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5×10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by

Klaus Bayreuther et al 1988. Though the proportion of f1 increased during the 3rd to 5th day there was finally decrease on the last day of observation. Similar changes were also seen in the proportion of f2. In case of f3 the peak was reached during 6th day. There was an increase in the proportion of f4, f6 and f7.

Permanent sample 9 **(GRAPH 22 & TABLE 31):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Klaus Bayreuther et al 1988. There was apparent decrease in f1 and f2. But f2 showed a increase during the 3rd and 4th day period. Rest of the subtypes showed increase in their respective proportion.

Permanent sample 12 **(GRAPH 23 & TABLE 32):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Klaus Bayreuther et al 1988. F1 and f2 showed apparently increase during the 2nd to 5th day. Likewise f3 and f5 showed a decrease during the same 2nd to 5th day. There was apparent increase in f4 and f7.

Stem Cells From Human Exfoliated Deciduous Tooth (Deciduous Tooth Sample)

Deciduous sample 1**(GRAPH 24 & TABLE 33):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 days

90 cells were randomly observed each day based on the subtype classification by Klaus Bayreuther et al 1988. There were increase in f1, f2, f3 and f7. With f1 reaching its peak during 3rd to 5th day, f2 reaching its peak value in 5th day and f3 reached the peak on the 4th day. f4 apparently showed no changes but it reduced during 4th day

Deciduous sample 2 **(GRAPH 25 & TABLE 34):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells /ml. Thereafter daily for 8 days 90 cells were randomly observed each day based on the subtype classification by Klaus Bayreuther et al 1988. There were increase in f1, f2, f4 and f5. The peak value of f2 reached its peak value during 3rd to 6th day. There were increase in the proportion of f6 and f7 during the log phase.

Deciduous sample 3 **(GRAPH 26 & TABLE 35):**

Cells between third and fourth passage were plated on three 60mm tissue culture plates at a concentration of 0.5 x 10^4 cells/ml. Thereafter daily for 8 consecutive days 90 cells were observed by random selection based on the subtype classification by Klaus Bayreuther et al 1988. There was a decrease in the proportion of f1, f2 and f3. There was a increase in the post mitotic population like the f5, f6 and f7.

Deciduous sample 6 **(GRAPH 27 & TABLE 36):**

Cells between third and fourth passage were plated on three 60mm tissue culture plated at a concentration of 5 x 10^3 cells/ ml. Thereafter daily for 8 consecutive days 90 cells were observed by random selection based on the

subtype classification by Klaus Bayreuther et al 1988. There was decrease in f2 and f3 with their peak value during the 2^{nd} to 4^{th} day. There was a increase in the f1, f4 , f5, f6 and f7.

A COMPARATIVE ANALYSIS BETWEEN DENTAL PULP STEM CELLS & STEM CELLS FROM EXFOLIATED DECIDUOUS TEETH SEEDING EFFICENCY AND POPULATION DOUBLING TIME (TABLE 41, 42 & 43)

There was no statistically significant difference in the seeding efficiency and population doubling time (PDT) between the dental pulp stem cells and stem cells from exfoliated deciduous teeth ($p>.05$)

F1:F2:F3 FIBROBLAST SUBPOPULATION RATIO (TABLE 40)

A comparative analysis of the sub types f1 f2 and f3 based on the Mollenhauer et al 1986 sub classification between the DPSCs and SHED, resulted in no significant difference between the two groups. This result was statistically insignificant irrespective of the subtypes.

Mitotic & Post mitotic Subpopulation Proportions (GRAPH 28, 29, 30,31,32,33,34,35,36 & TABLE 37, 38, 39)

According to the Klaus Bayreuther et al 1988 subtypes f1, f2 and f3 were together considered to mitotic and f4, f5, f6 & f7 together constituted the post mitotic subpopulations. It was observed that irrespective of the samples from both deciduous and permanent tooth there existed a negative correlation between the mitotic and post mitotic subpopulation that was statistically significant.

Further comparing the mitotic and post mitotic subpopulation of both Dental pulp Stem cells and Stem cells from exfoliated deciduous teeth we observed that there existed no similarity between the two groups. This was statistically insignificant ($p>0.05$).

Expression of CD 106 or Vascular Cell Adhesion Molecule (VCAM) and STRO1 between Dental Pulp Stem cells (DPSC) & Stem cells from Human Exfoliated Deciduous teeth (SHED)

The stem cells isolated and cultured from the dental pulp of permanent and deciduous teeth did not express STRO1 and CD 106. But the controls considered lymphnodes for CD106 and Osteosarcom cells for STRO1 had the expression. **[TABLES 44 & 45] [FIGURES 19 &20]**

Tables & Graphs

GRAPH 1: GROWTH CURVE OF PERMANENT SAMPLE 2

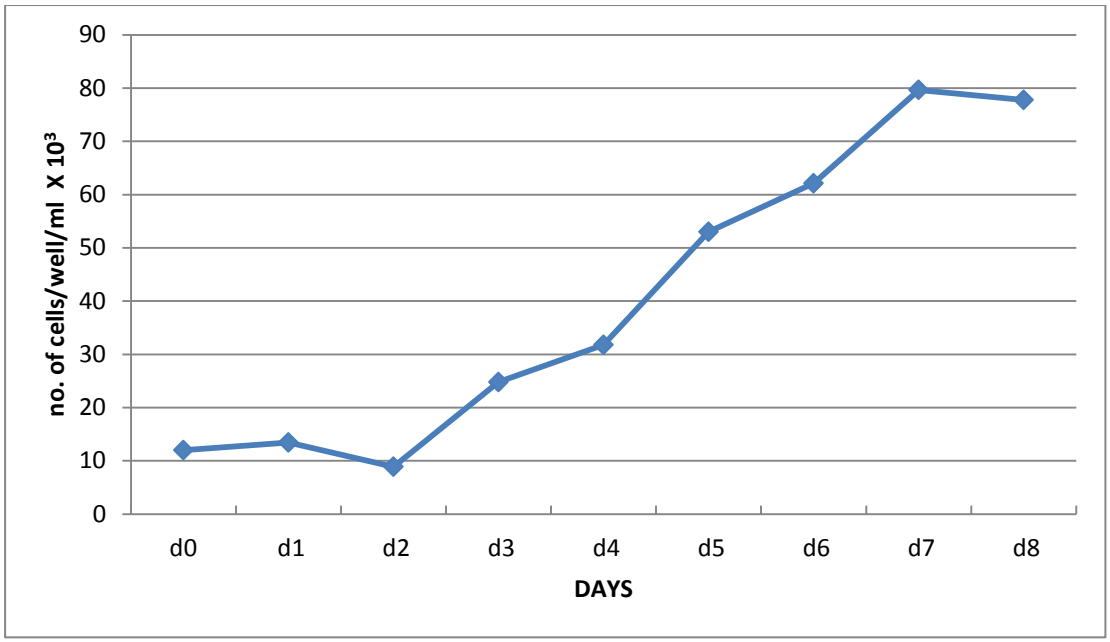


TABLE 1: GROWTH CURVE DERIVATIVES OF PERMANENT SAMPLE 2

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	10.93	8.71	43.92	112
1	13.44				
2	8.89				
3	24.78				
4	31.78				
5	53				
6	62.11				
7	79.66				
8	77.77				

GRAPH 2: GROWTH CURVE PERMANENT SAMPLE 3

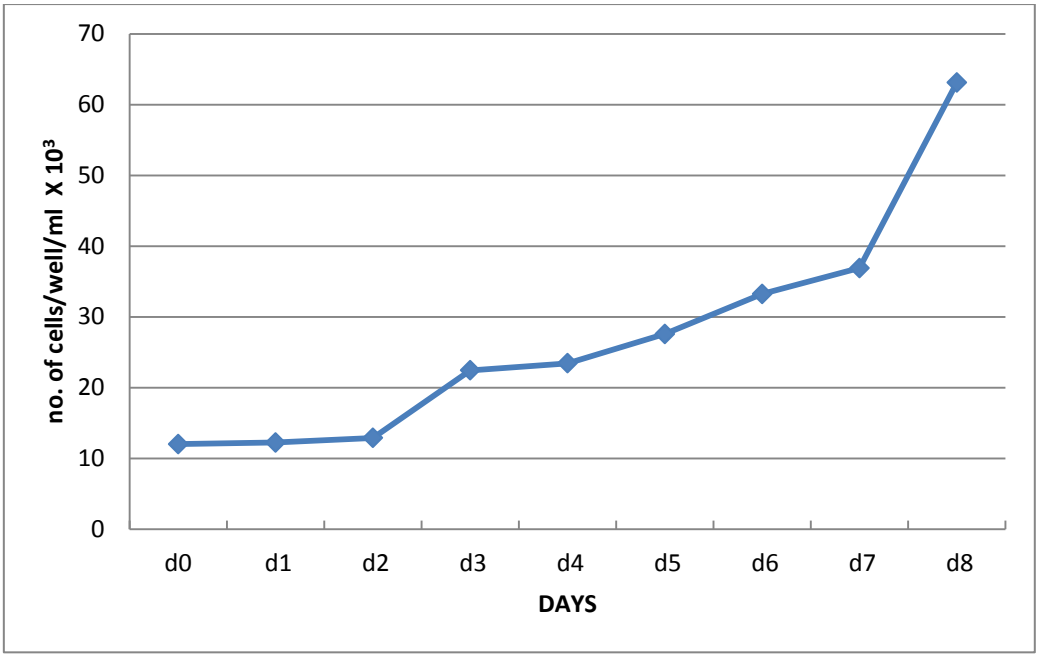


TABLE 2: GROWTH CURVE DERIVATIVES OF PERMANENT SAMPLE 3

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	4.29	3.36	111.99	101.83
1	12.22				
2	12.89				
3	22.44				
4	23.44				
5	27.56				
6	33.22				
7	36.88				
8	63.11				

GRAPH 3: GROWTH CURVE OF PERMANENT SAMPLE 6

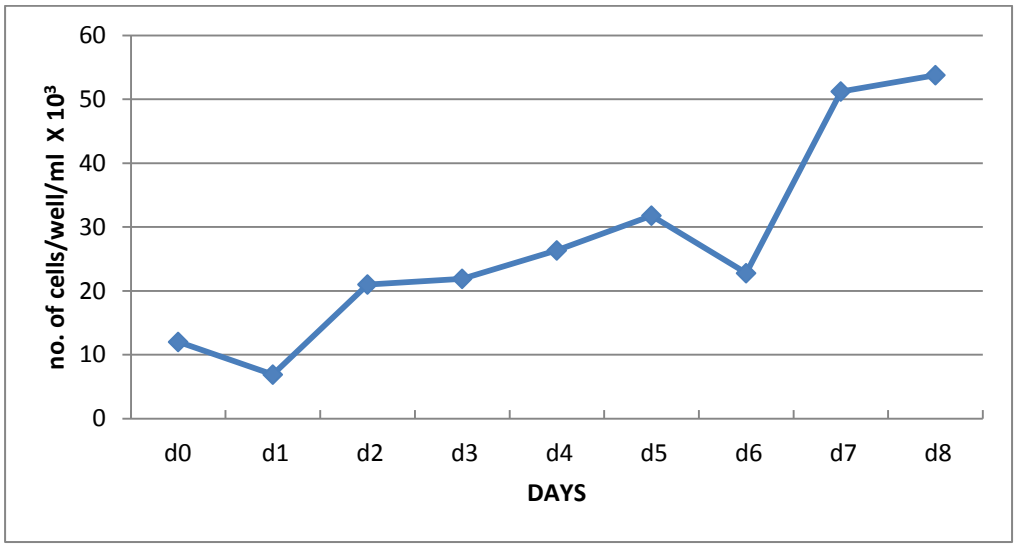


TABLE 3: GROWTH CURVE DERIVATIVES OF PERMANENT SAMPLE 6

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	3.32	3.39	144.62	57.41
1	6.889				
2	21				
3	21.889				
4	26.333				
5	31.778				
6	22.77				
7	51.22				
8	53.778				

GRAPH 4: GROWTH CURVE OF PERMANENT SAMPLE 9

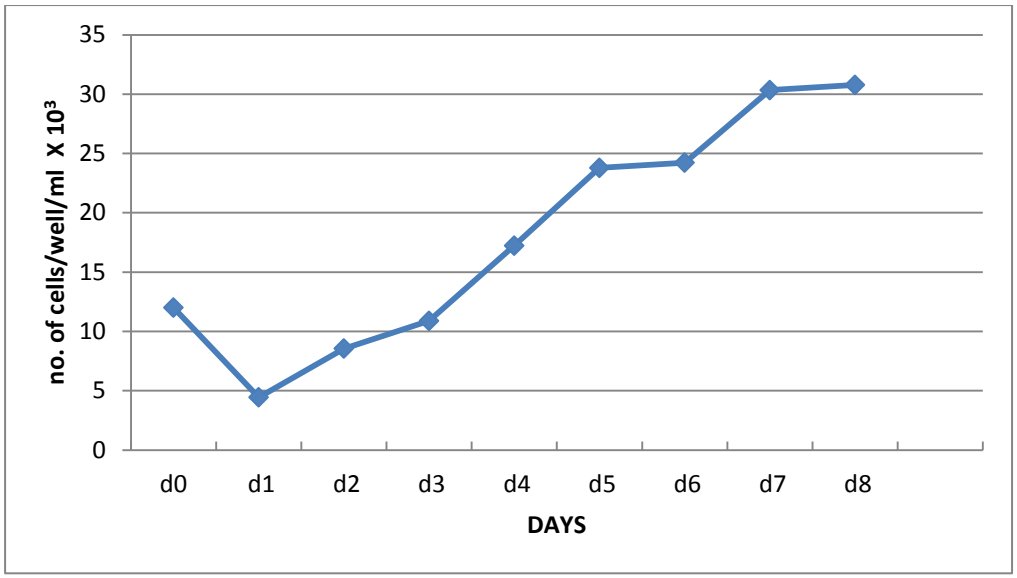


TABLE 4: GROWTH CURVE DERIVATIVES OF PERMANENT SAMPLE 9

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	4.31	3.35	111.32	37
1	4.44				
2	8.55				
3	10.88				
4	17.22				
5	23.78				
6	24.22				
7	30.33				
8	30.78				

GRAPH 5: GROWTH CURVE OF PERMANENT SAMPLE 12

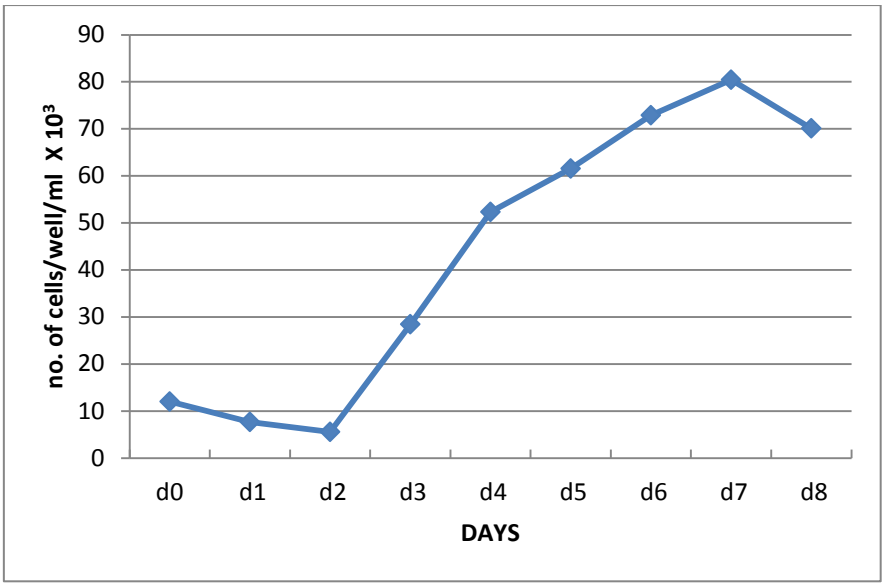


TABLE 5: GROWTH CURVE DERIVATIVES OF PERMANENT SAMPLE 12

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	14.8	11.61	32.43	63.89
1	7.67				
2	5.56				
3	28.44				
4	52.33				
5	61.56				
6	72.89				
7	80.44				
8	70.11				

GRAPH 6: GROWTH CURVE OF DECIDUOUS SAMPLE 1

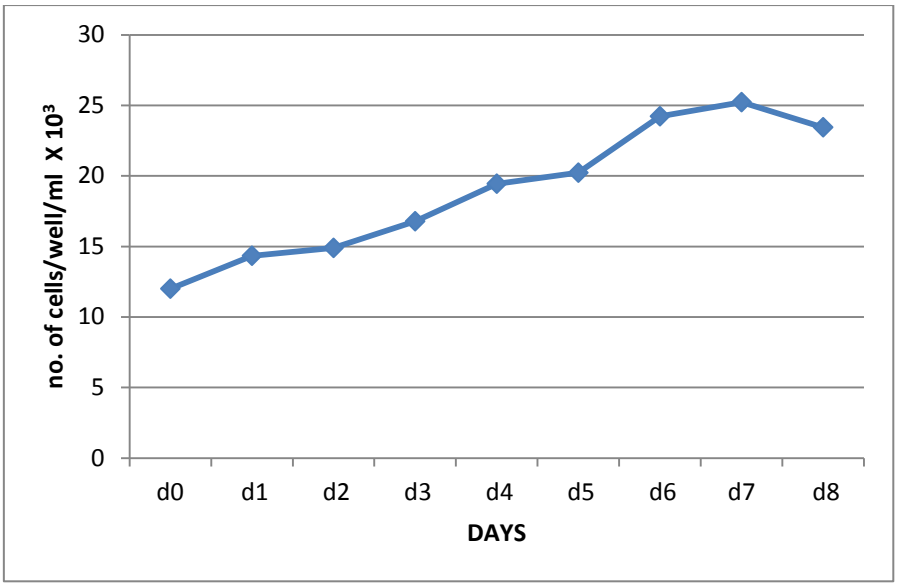


TABLE 6: GROWTH CURVE DERIVATIVES OF DECIDUOUS SAMPLE 1

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	1.95	2.03	246.79	119.42
1	14.33				
2	14.89				
3	16.78				
4	19.44				
5	20.22				
6	24.22				
7	25.22				
8	23.44				

GRAPH 7: GROWTH CURVE OF DECIDUOUS SAMPLE 2

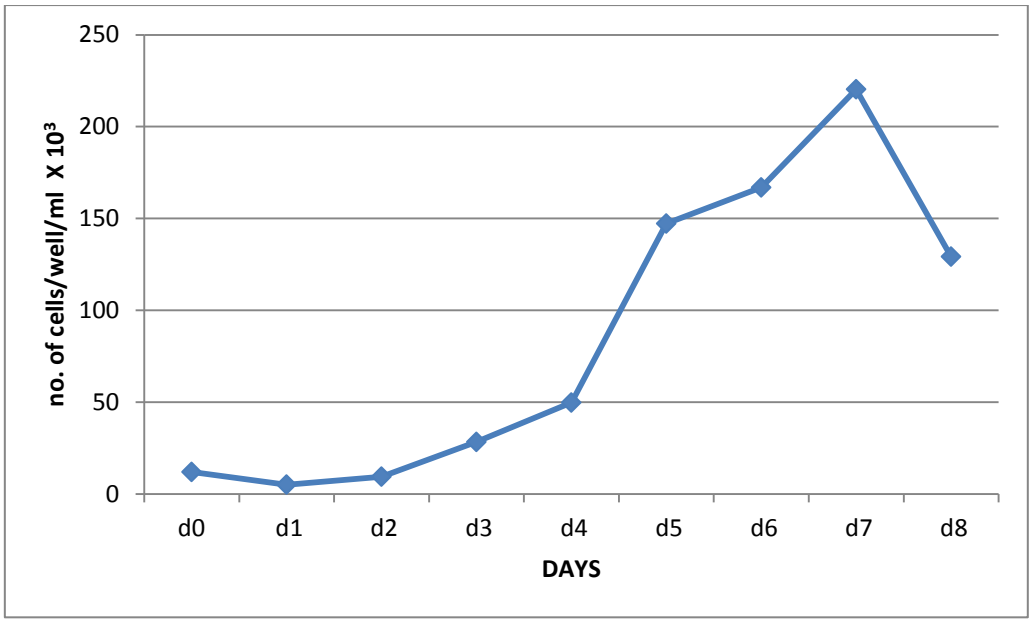


TABLE 7: GROWTH CURVE DERIVATIVES OF DECIDUOUS SAMPLE 2

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	35.53	29.07	13.51	42.59
1	5.11				
2	9.44				
3	28.33				
4	49.78				
5	147.22				
6	166.88				
7	220.33				
8	129.22				

GRAPH 8: GROWTH CURVE OF DECIDUOUS SAMPLE 3

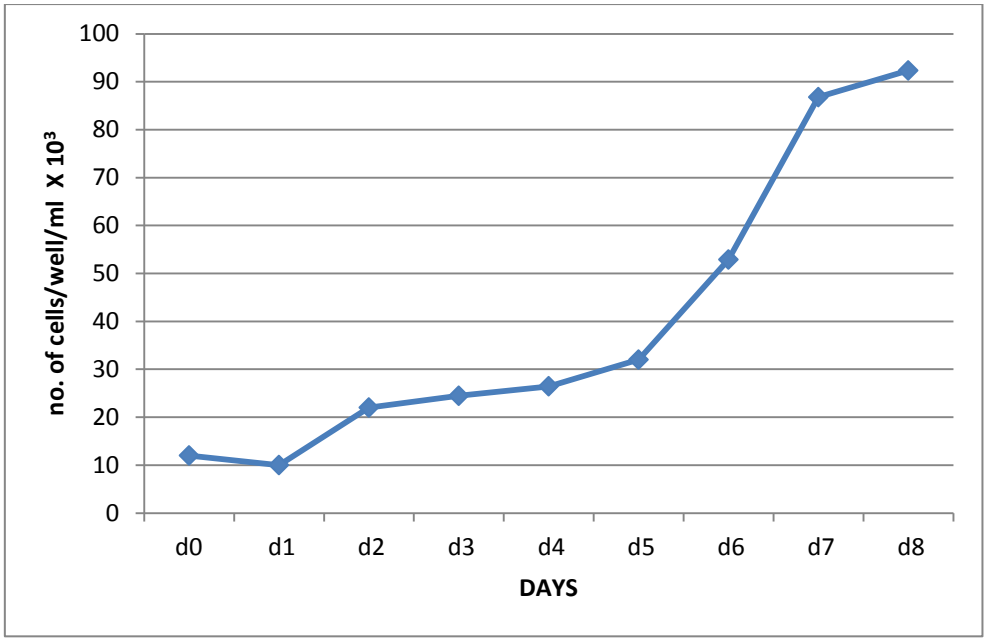


TABLE 8: GROWTH CURVE DERIVATIVES OF DECIDUOUS SAMPLE 3

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	7.04	5.80	68.18	83.33
1	10				
2	22				
3	24.55				
4	26.44				
5	32				
6	52.88				
7	86.78				
8	92.33				

GRAPH 9: GROWTH CURVE OF DECIDOUS SAMPLE 6

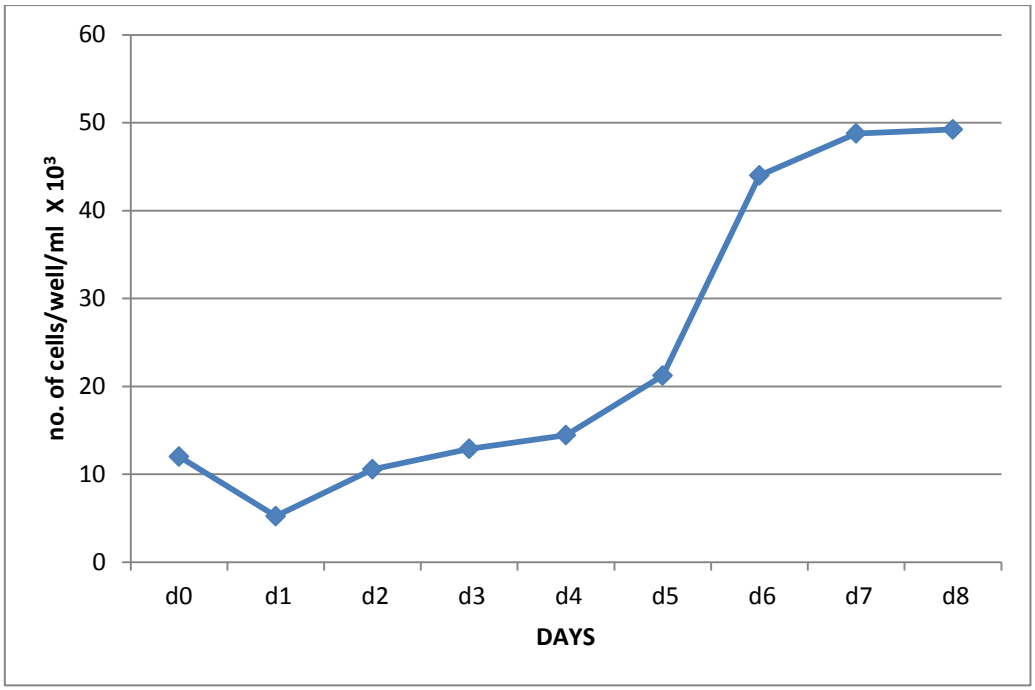


TABLE 9: GROWTH CURVE DERIVATIVES OF DECIDUOUS SAMPLE 6

DAYS	Cell count /well/ml X 10^3	Slope	Standard error	Population doubling time in hours	Seeding efficiency %
0	12	6.49	5.61	73.87	43.5
1	5.22				
2	10.56				
3	12.89				
4	14.44				
5	21.22				
6	44				
7	48.78				
8	49.22				

GRAPH 10 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 2

f1 –f3 [MOLLENHAUER et al 1986]

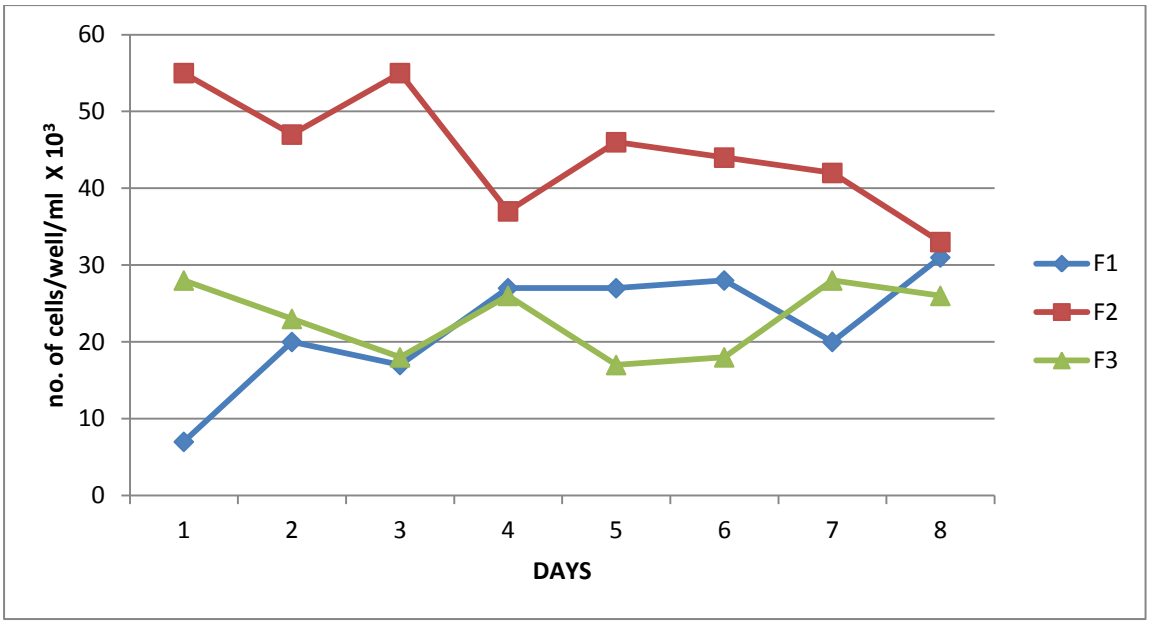


TABLE 10 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF PERMANENT TOOTH SAMPLE 2 FOR 8 DAYS

DAYS	F1	F2	F3
1	7	55	28
2	20	47	23
3	17	55	18
4	27	37	26
5	27	46	17
6	28	44	18
7	20	42	28
8	31	33	26

TABLE 11 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF PERMANENT TOOTH SAMPLE 2

SUBPOPULATION	CORRELATION COEFFICIENT (r)	p-value
f1	.75	.031*
f2	.76	.028*
f3	.012	.977

*- statistically significant at 5% level

GRAPH 11 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 3

f1 –f3 [MOLLENHAUER et al 1986]

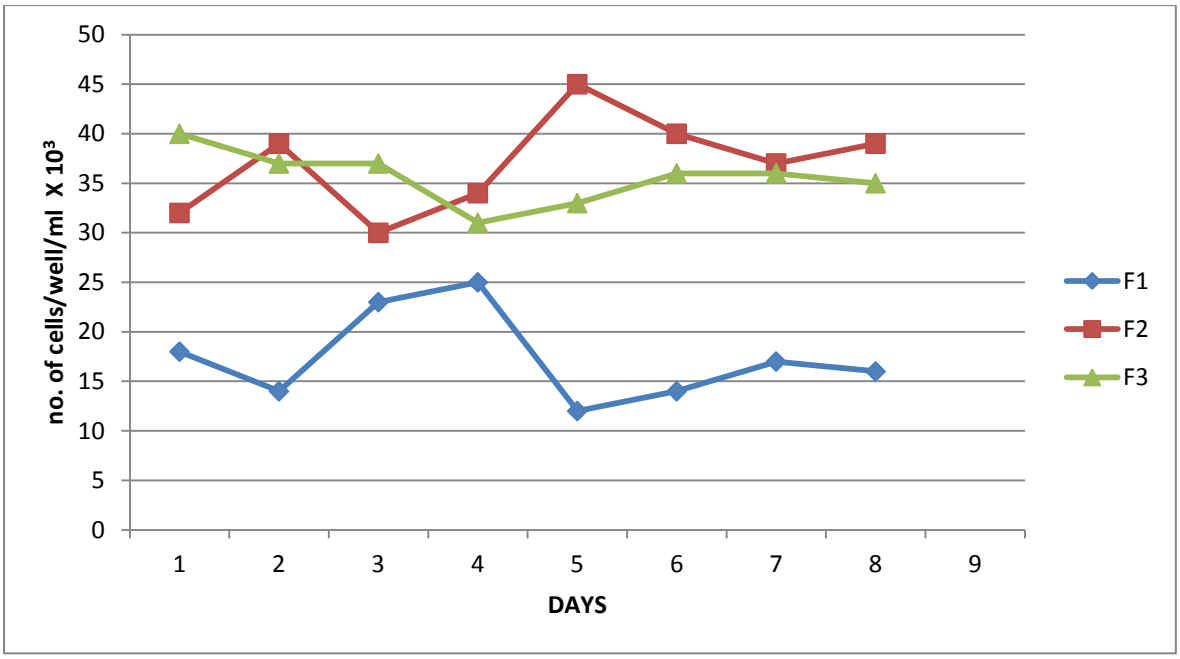


TABLE 12 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF PERMANENT TOOTH SAMPLE 3 FOR 8 DAYS

DAYS	F1	F2	F3
1	18	32	40
2	14	39	37
3	23	30	37
4	25	39	31
5	12	45	33
6	14	40	36
7	17	37	36
8	16	39	35

TABLE 13 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF PERMANENT TOOTH SAMPLE 3

SUBPOPULATION	CORRELATION COEFFICIENT (r)	P value
f1	.25	.55
f2	.48	.23
f3	.44	.28

GRAPH 12 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 6

f1 –f3 [MOLLENHAUER et al 1986]

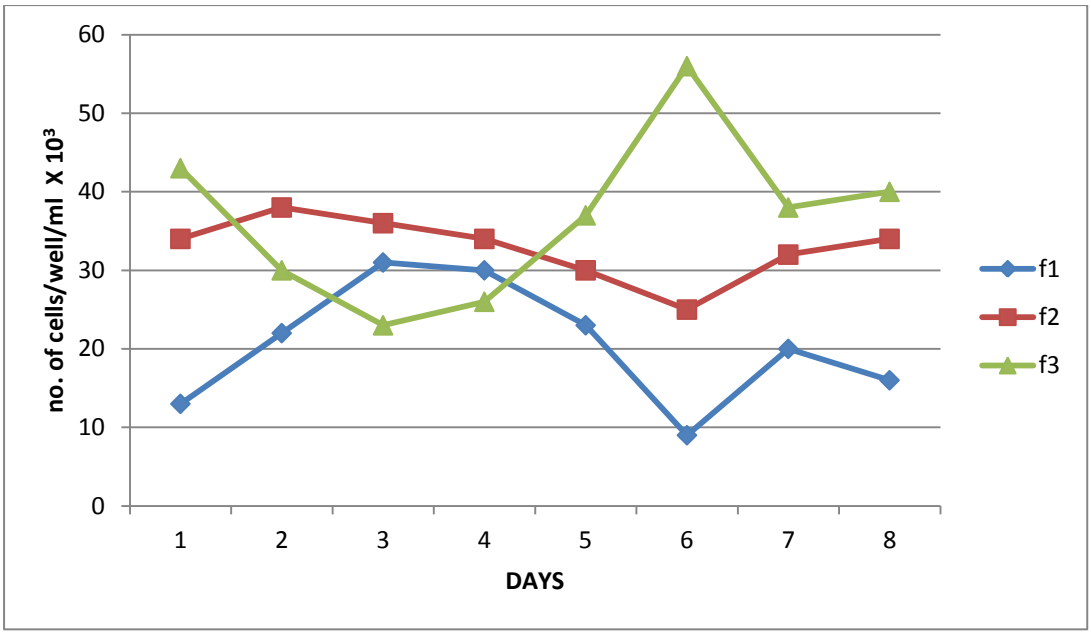


TABLE 14 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF PERMANENT TOOTH SAMPLE 6 FOR 8 DAYS

DAYS	F1	F2	F3
1	13	34	43
2	22	38	30
3	31	36	23
4	30	34	26
5	23	30	37
6	9	25	56
7	20	32	38
8	16	34	40

TABLE 15 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF PERMANENT TOOTH SAMPLE 6

SUBPOPULATION	CORRELATION COEFFICIENT (r)	P - value
f1	.23	.58
f2	.49	.22
f3	.36	.38

GRAPH 13 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 9

f1 –f3 [MOLLENHAUER et al 1986]

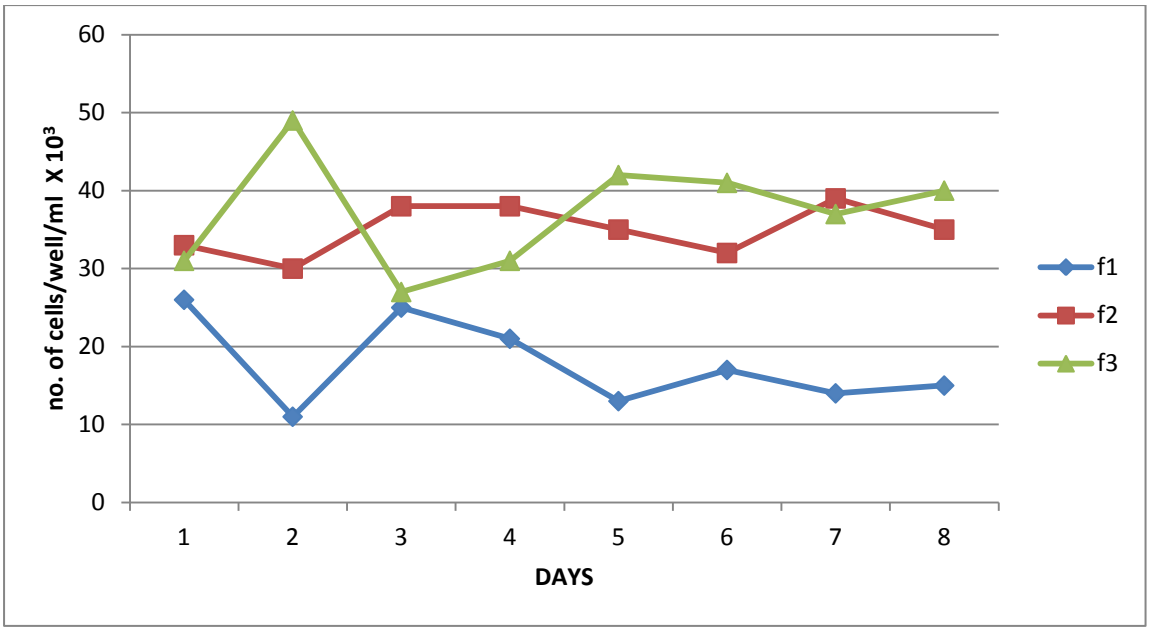


TABLE 16 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF PERMANENT TOOTH SAMPLE 9 FOR 8 DAYS

DAYS	F1	F2	F3
1	26	33	31
2	11	30	49
3	25	38	27
4	21	38	31
5	13	35	42
6	17	32	41
7	14	39	37
8	15	35	40

TABLE 17 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF PERMANENT TOOTH SAMPLE 9

SUBPOPULATION	CORRELATION COEFFICIENT (r)	P- Value
f1	.49	.22
f2	.35	.40
f3	.23	.59

GRAPH 14 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 12

f1 –f3 [MOLLENHAUER et al 1986]

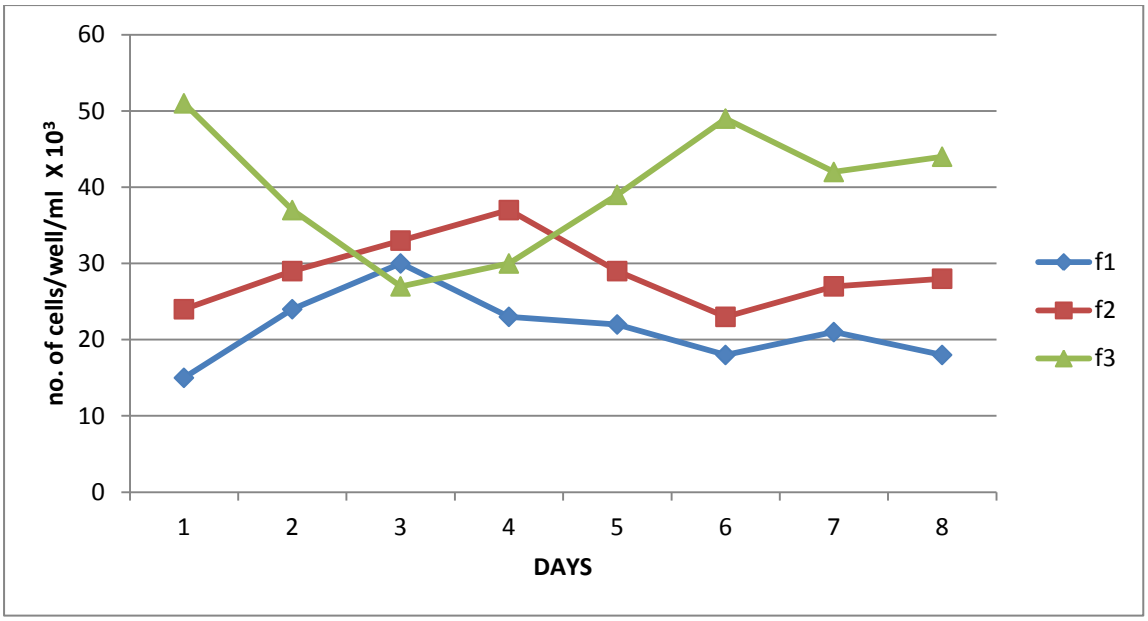


TABLE 18 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF PERMANENT TOOTH SAMPLE 12 FOR 8 DAYS

DAYS	F1	F2	F3
1	15	24	51
2	24	29	37
3	30	33	27
4	23	37	30
5	22	29	39
6	18	23	49
7	21	27	42
8	18	28	44

TABLE 19 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF PERMANENT TOOTH SAMPLE 12

SUBPOPULATION	CORRELATION COEFFICIENT (r)	P Value
f1	.19	.64
f2	.13	.76
f3	.18	.68

GRAPH 15 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 1

f1 –f3 [MOLLENHAUER et al 1986]

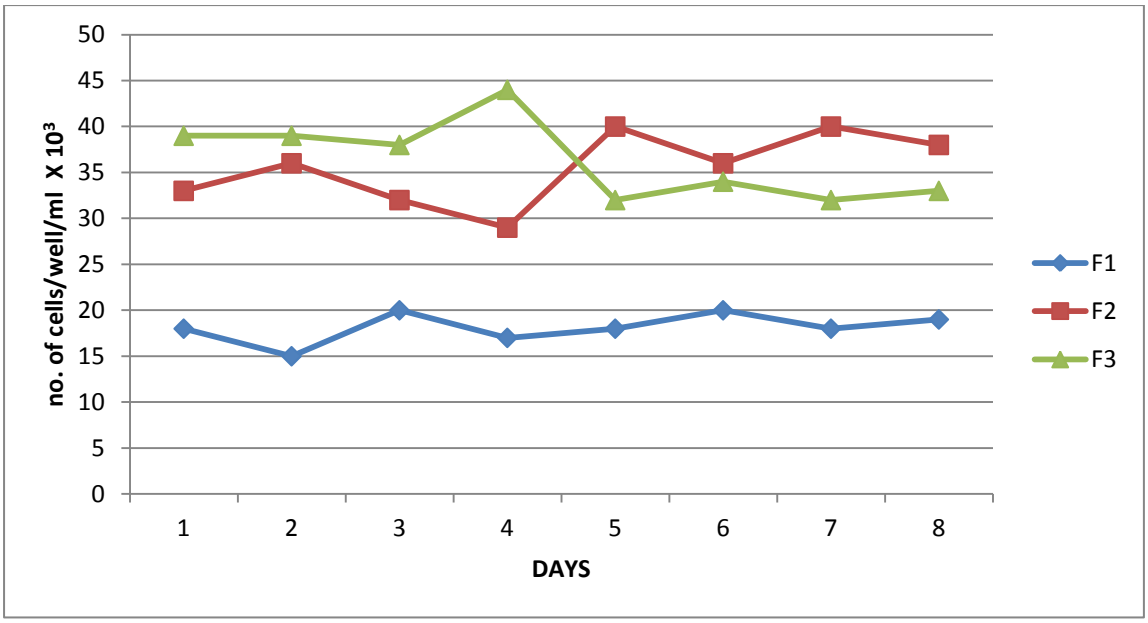


TABLE 20 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF DECIDUOUS TOOTH SAMPLE 1 FOR 8 DAYS

DAYS	F1	F2	F3
1	18	33	39
2	15	36	39
3	20	32	38
4	17	29	44
5	18	40	32
6	20	36	34
7	18	40	32
8	19	38	33

TABLE 21 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF DECIDUOUS TOOTH SAMPLE 1

SUBPOPULATION	CORRELATION COEFFICIENT (r)	p-value
f1	.41	.32
f2	.58	.13
f3	.68	.06

GRAPH 16 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 2

f1 –f3 [MOLLENHAUER et al 1986]

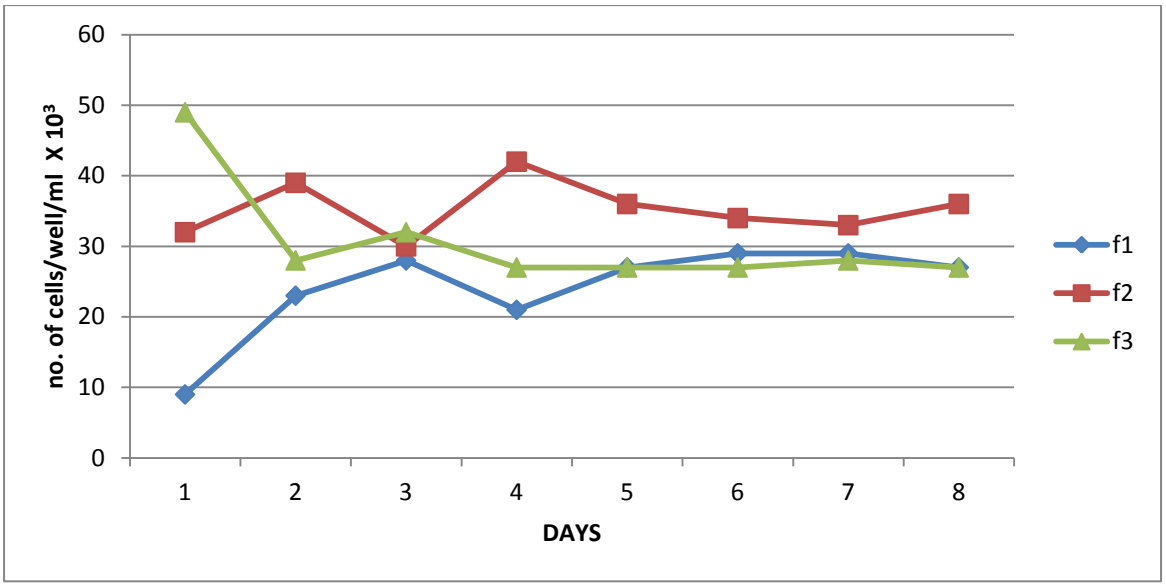


TABLE 22 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF DECIDUOUS TOOTH SAMPLE 2 FOR 8 DAYS

DAYS	F1	F2	F3
1	9	32	49
2	23	39	28
3	28	30	32
4	21	42	27
5	27	36	27
6	29	34	27
7	29	33	28
8	27	36	27

TABLE 23 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF DECIDUOUS TOOTH SAMPLE 2

SUBPOPULATION	CORRELATION COEFFICIENT (r)	P value
f1	.84	.009*
f2	.03	.94
f3	.65	.08

*-statistically significant at 5% level

GRAPH 17 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 3

f1 –f3 [MOLLENHAUER et al 1986]

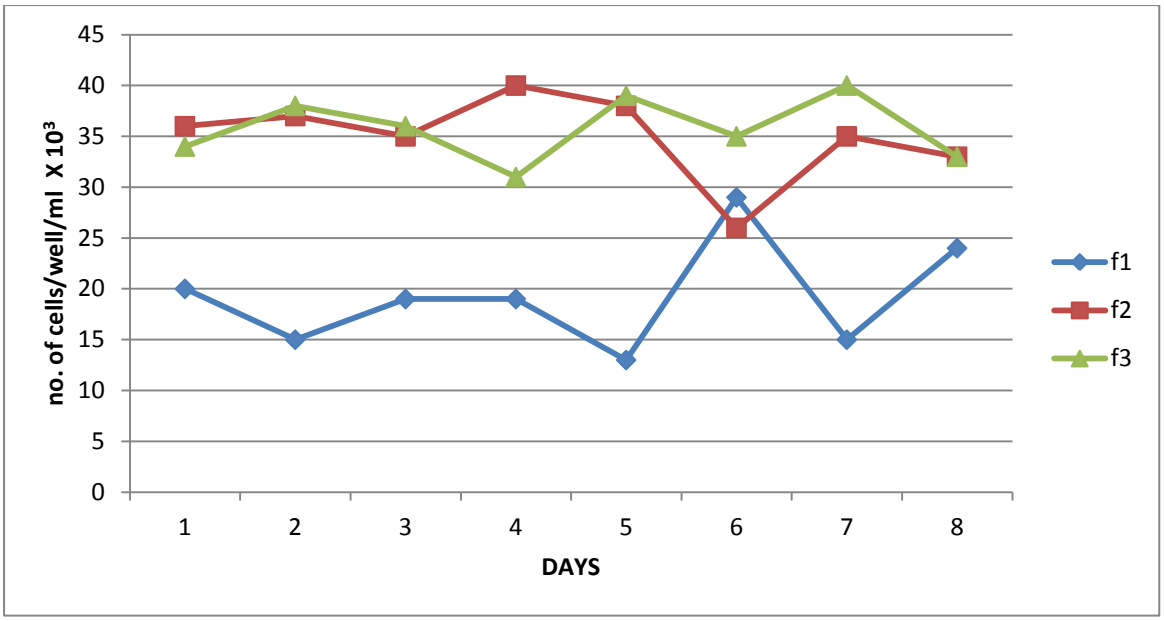


TABLE 24 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF DECIDUOUS TOOTH SAMPLE 3 FOR 8 DAYS

DAYS	F1	F2	F3
1	20	36	34
2	15	37	38
3	19	35	36
4	19	40	31
5	13	38	39
6	29	26	35
7	15	35	40
8	24	33	33

TABLE 25 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF DECIDUOUS TOOTH SAMPLE 3

SUBPOPULATION	CORRELATION COEFFICIENT (r)	p-value
f1	.29	.49
f2	.42	.31
f3	.08	.86

GRAPH 18 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 6

f1 –f3 [MOLLENHAUER et al 1986]

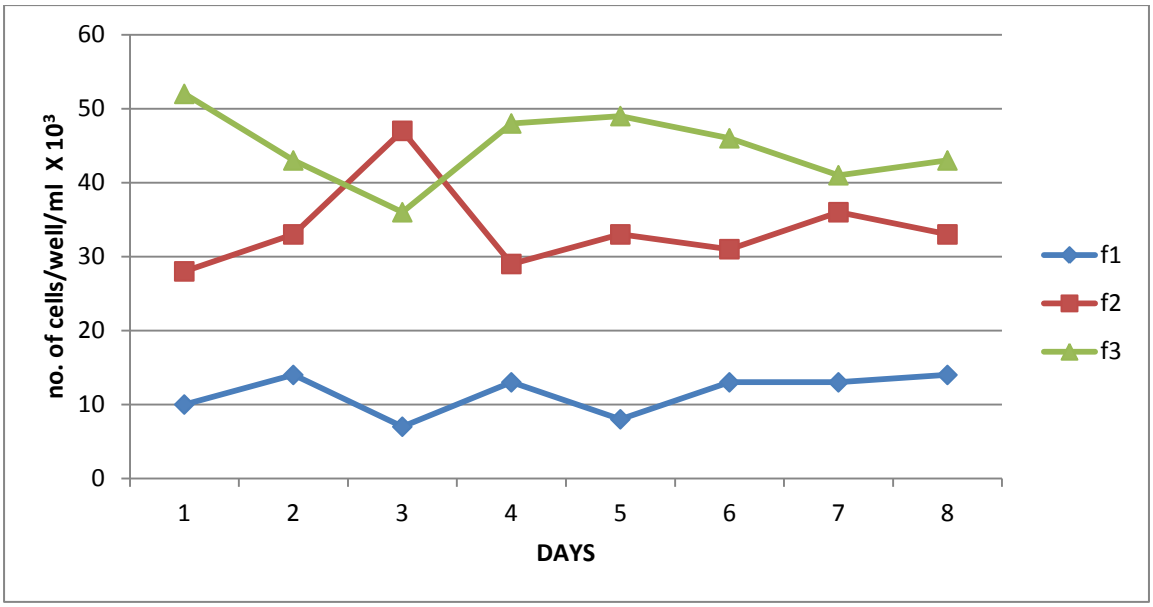


TABLE 26 - SUB-POPULATION PROPORTIONS IN 90 CELLS OF DECIDUOUS TOOTH SAMPLE 6 FOR 8 DAYS

DAYS	F1	F2	F3
1	10	28	52
2	14	33	43
3	7	47	36
4	13	29	48
5	8	33	49
6	13	31	46
7	13	36	41
8	14	33	43

TABLE 27 – CORRELATION COEFFICIENT OF THE SUB-POPULATIONS OF DECIDUOUS TOOTH SAMPLE 6

SUBPOPULATION	CORRELATION COEFFICIENT (r)	p- value
f1	.38	.36
f2	.03	.95
f3	.24	.56

GRAPH 19 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 2

f1 to f7 [KLAUS BAYREUTHER et al 1988]

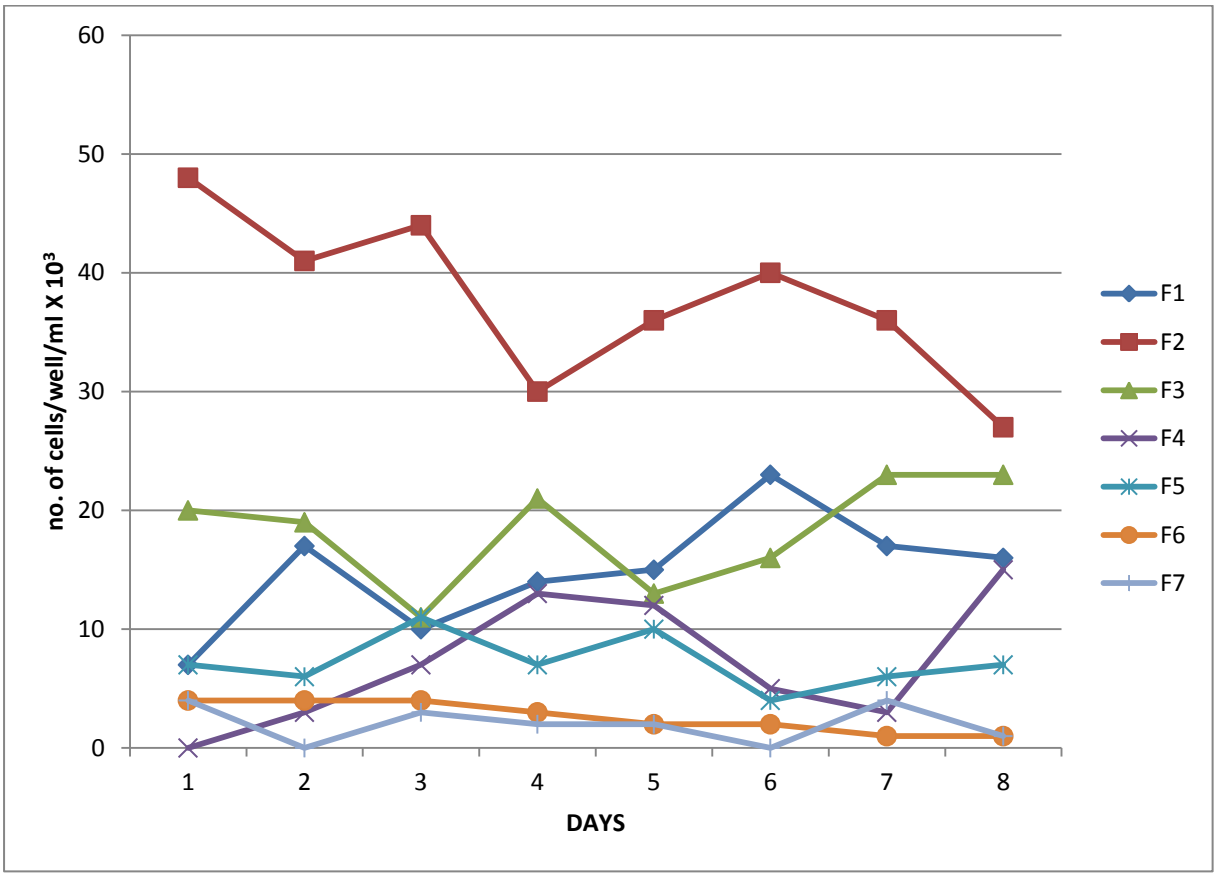


TABLE 28 – SUBPOPULATION PROPORTION OF 90 CELLS OF PERMANENT TOOTH SAMPLE 2 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	7	48	20	-	7	4	4
2	17	41	19	3	6	4	-
3	10	44	11	7	11	4	3
4	14	30	21	13	7	3	2
5	15	36	13	12	10	2	2
6	23	40	16	5	4	2	-
7	17	36	23	3	6	1	4
8	16	27	23	15	7	1	1

GRAPH 20 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 3

f1 to f7 [KLAUS BAYREUTHER et al 1988]

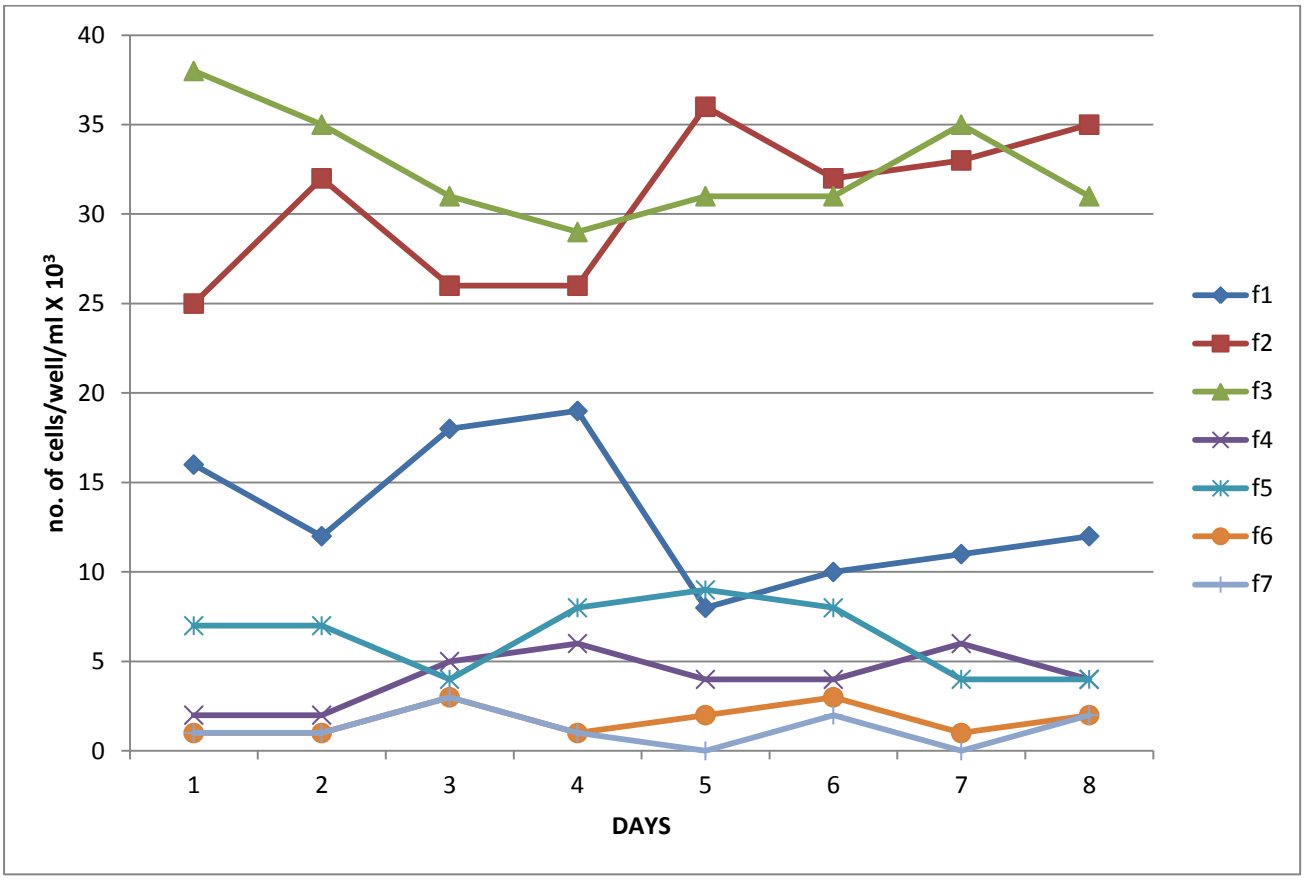


TABLE 29 – SUBPOPULATION PROPORTION OF 90 CELLS OF PERMANENT TOOTH SAMPLE 3 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	16	25	38	2	7	1	1
2	12	32	35	2	7	1	1
3	18	26	31	5	4	3	3
4	19	26	29	6	8	1	1
5	8	36	31	4	9	2	-
6	10	32	31	4	8	3	2
7	11	33	35	6	4	1	-
8	12	35	31	4	4	2	2

GRAPH 21 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 6

f1 to f7 [KLAUS BAYREUTHER et al 1988]

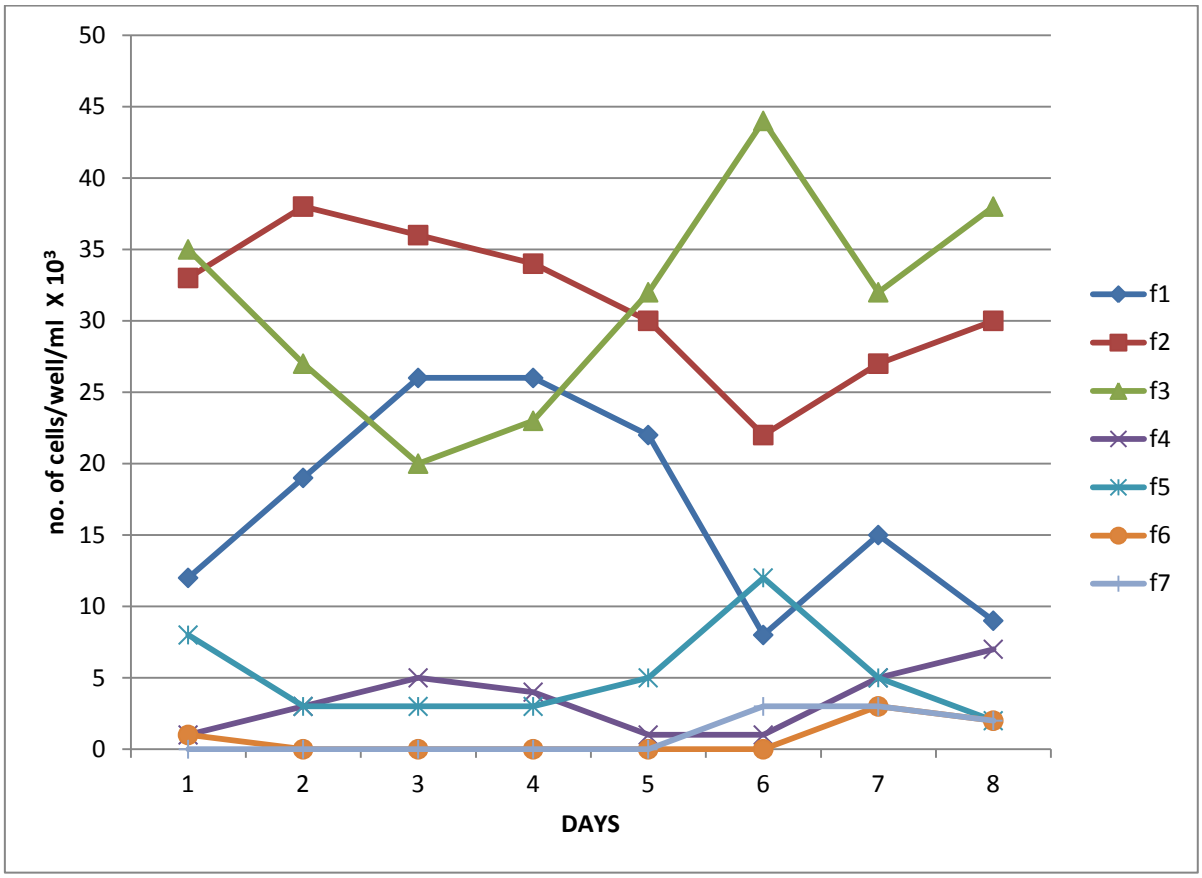


TABLE 30 – SUBPOPULATION PROPORTION OF 90 CELLS OF PERMANENT TOOTH SAMPLE 6 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	12	33	35	1	8	1	-
2	19	38	27	3	3	-	-
3	26	36	20	5	3	-	-
4	26	34	23	4	3	-	-
5	22	30	32	1	5	-	-
6	8	22	44	1	12	-	3
7	15	27	32	5	5	3	3
8	9	30	38	7	2	2	2

GRAPH 22 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 9

f1 to f7 [KLAUS BAYREUTHER et al 1988]

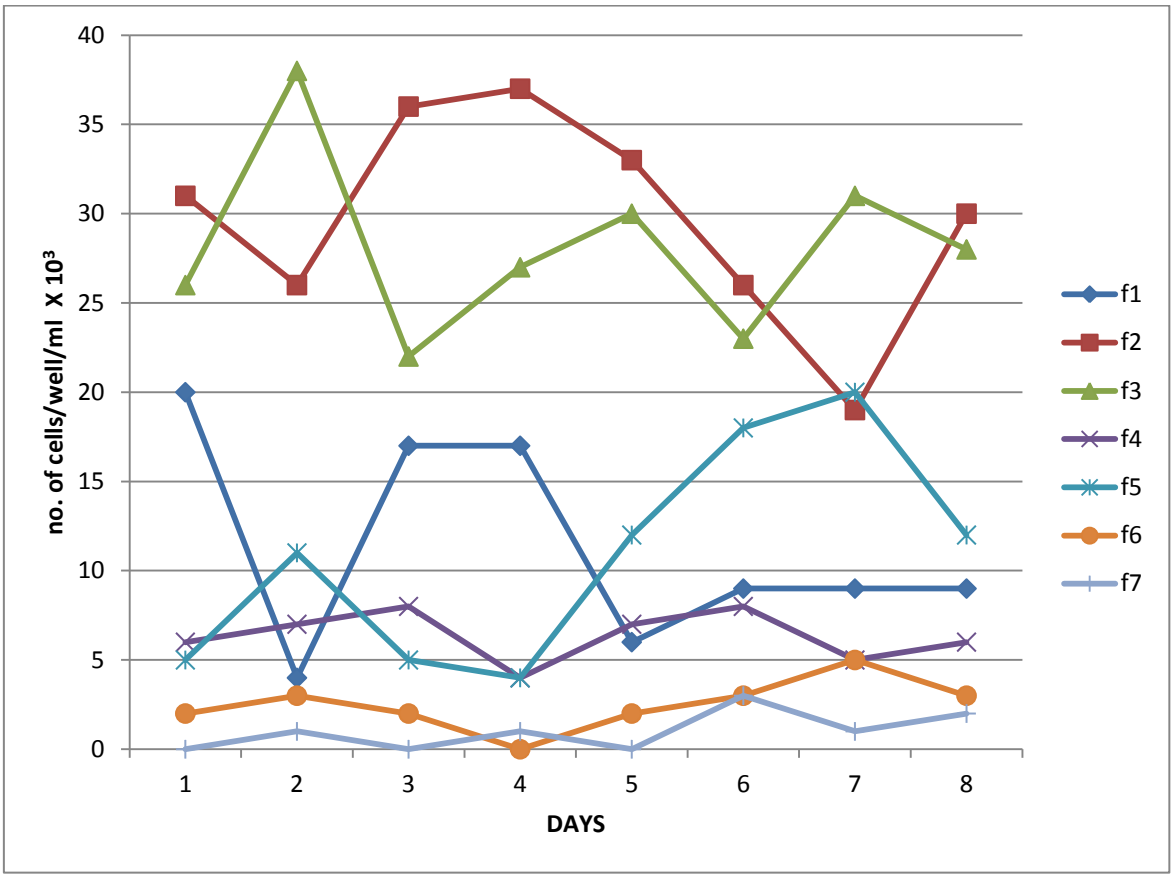


TABLE 31 – SUBPOPULATION PROPORTION OF 90 CELLS OF PERMANENT TOOTH SAMPLE 9 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	20	31	26	6	5	2	-
2	4	26	38	7	11	3	1
3	17	36	22	8	5	2	-
4	17	37	27	4	4	-	1
5	6	33	30	7	12	2	-
6	9	26	23	8	18	3	3
7	9	19	31	5	20	5	1
8	9	30	28	6	12	3	2

GRAPH 23 – SUBPOPULATION ANALYSIS OF PERMANENT TOOTH SAMPLE 12

f1 to f7 [KLAUS BAYREUTHER et al 1988]

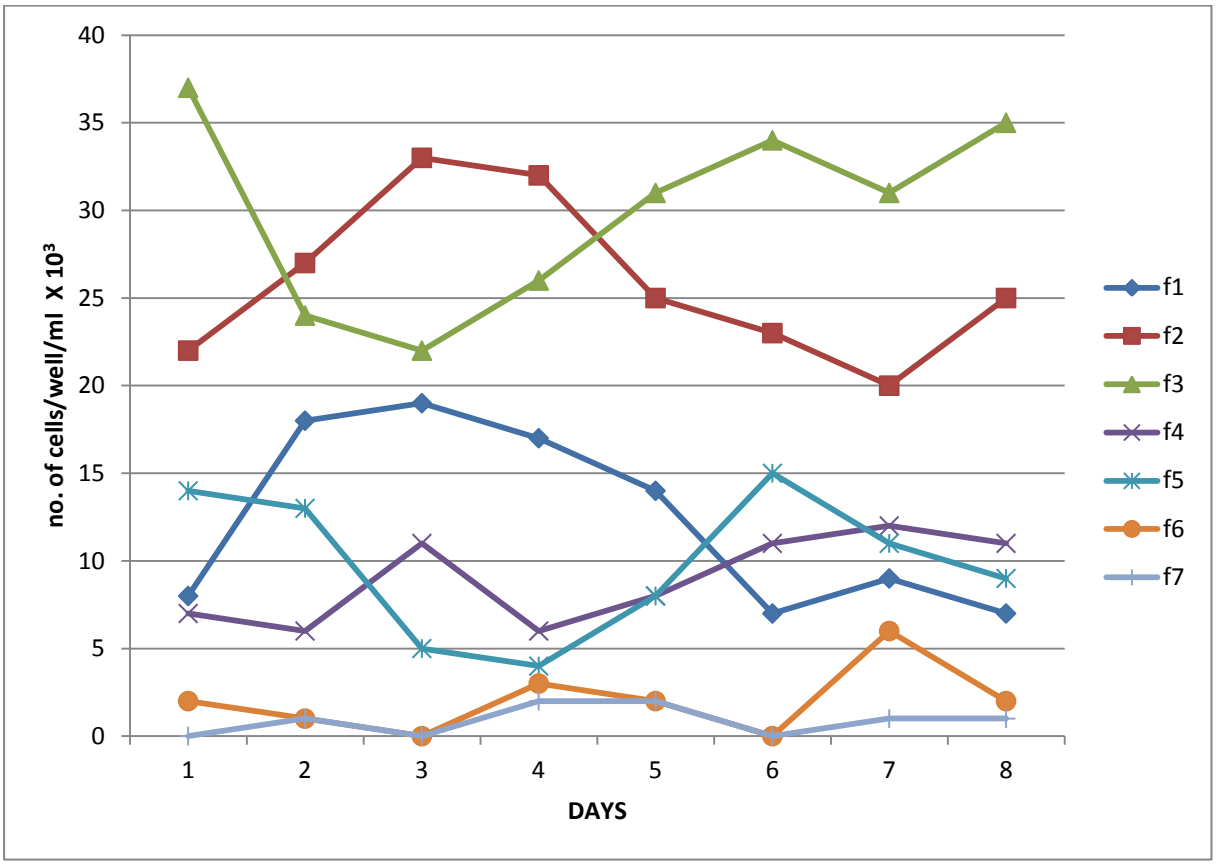


TABLE 32 – SUBPOPULATION PROPORTION OF 90 CELLS OF PERMANENT TOOTH SAMPLE 12 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	8	22	37	7	14	2	-
2	18	27	24	6	13	1	1
3	19	33	22	11	5	-	-
4	17	32	26	6	4	3	2
5	14	25	31	8	8	2	2
6	7	23	34	11	15	-	-
7	9	20	31	12	11	6	1
8	7	25	35	11	9	2	1

GRAPH 24 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 1

f1 to f7 [KLAUS BAYREUTHER et al 1988]

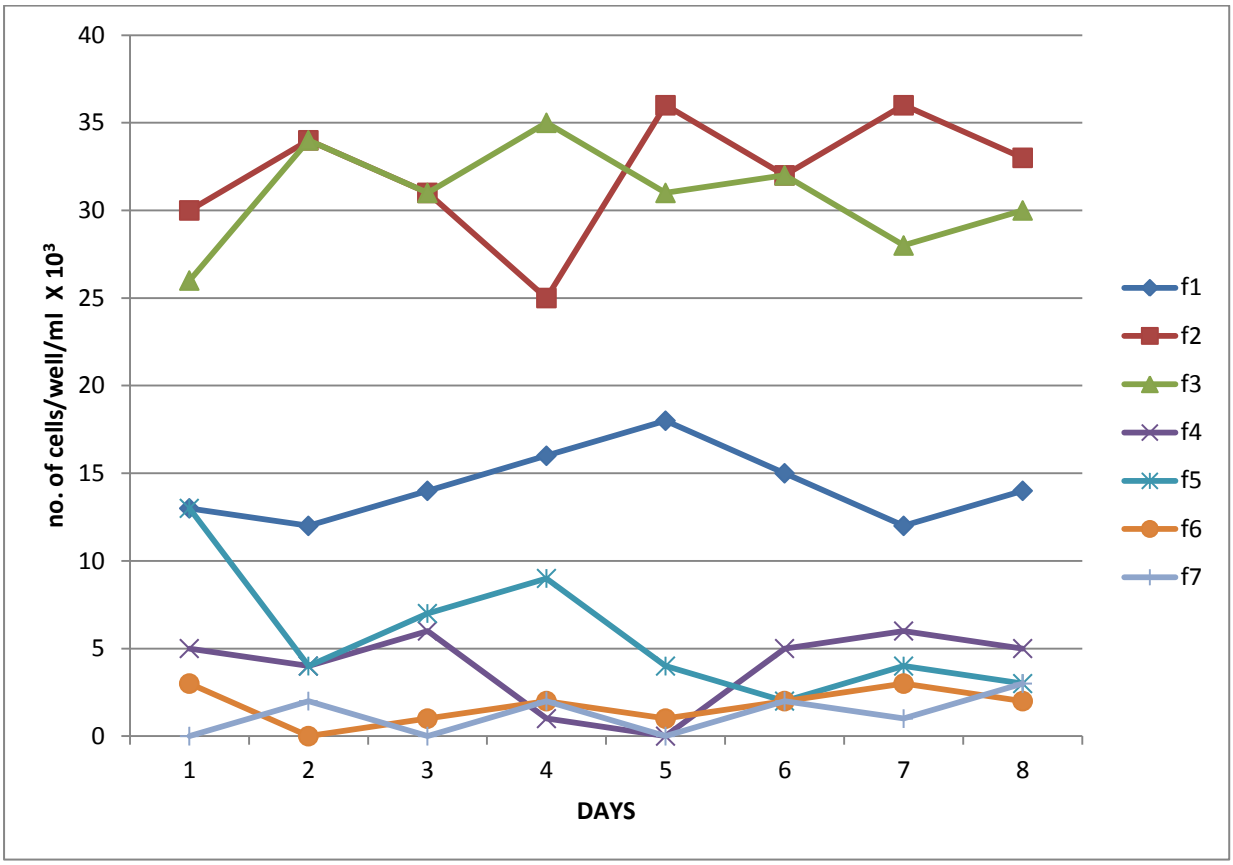


TABLE 33 – SUBPOPULATION PROPORTION OF 90 CELLS OF DECIDUOUS TOOTH SAMPLE 1 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	13	30	26	5	13	3	-
2	12	34	34	4	4	-	2
3	14	31	31	6	7	1	-
4	16	25	35	1	9	2	2
5	18	36	31	-	4	1	-
6	15	32	32	5	2	2	2
7	12	36	28	6	4	3	1
8	14	33	30	5	3	2	3

GRAPH 25 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 2

f1 to f7 [KLAUS BAYREUTHER et al 1988]

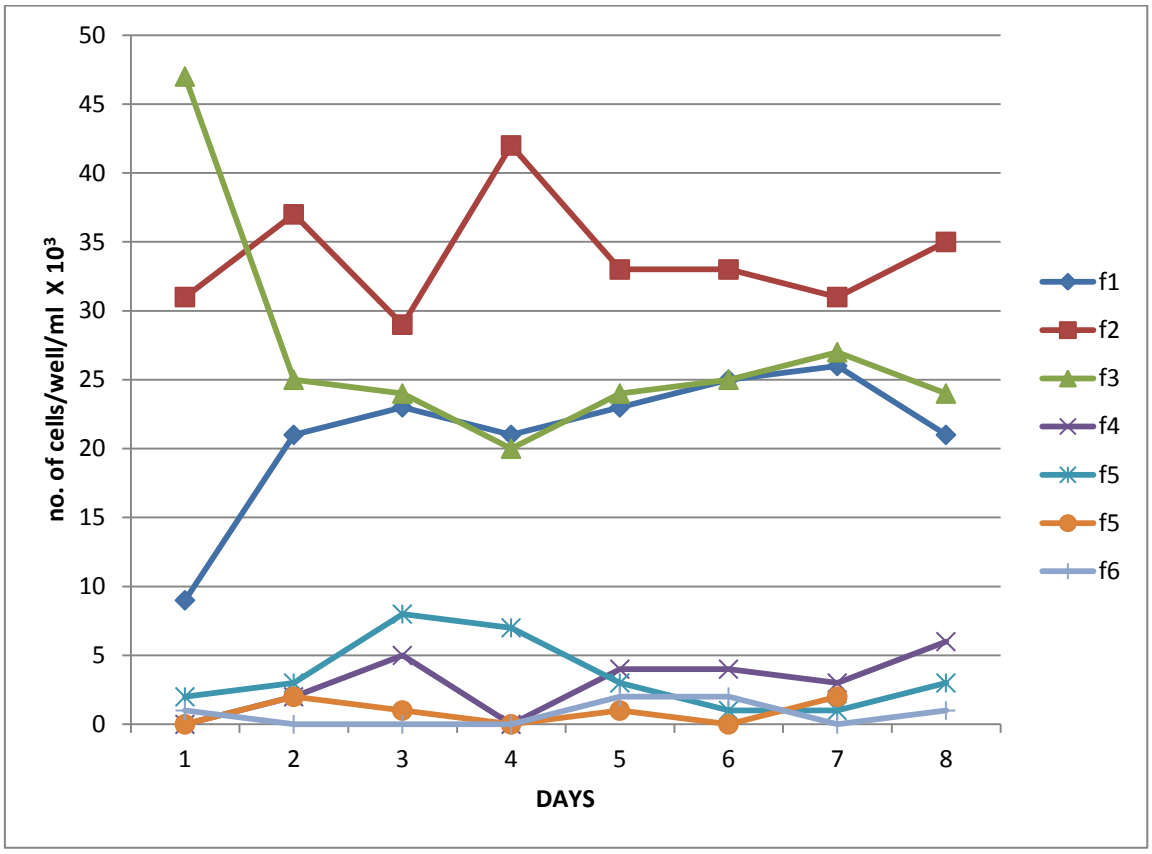


TABLE 34 – SUBPOPULATION PROPORTION OF 90 CELLS OF DECIDUOUS TOOTH SAMPLE 2 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	9	31	47	-	2	-	1
2	21	37	25	2	3	2	-
3	23	29	24	5	8	1	-
4	21	42	20	-	7	-	-
5	23	33	24	4	3	1	2
6	25	33	25	4	1	-	2
7	26	31	27	3	1	2	-
8	21	35	24	6	3	-	1

GRAPH 26 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 3

f1 to f7 [KLAUS BAYREUTHER et al 1988]

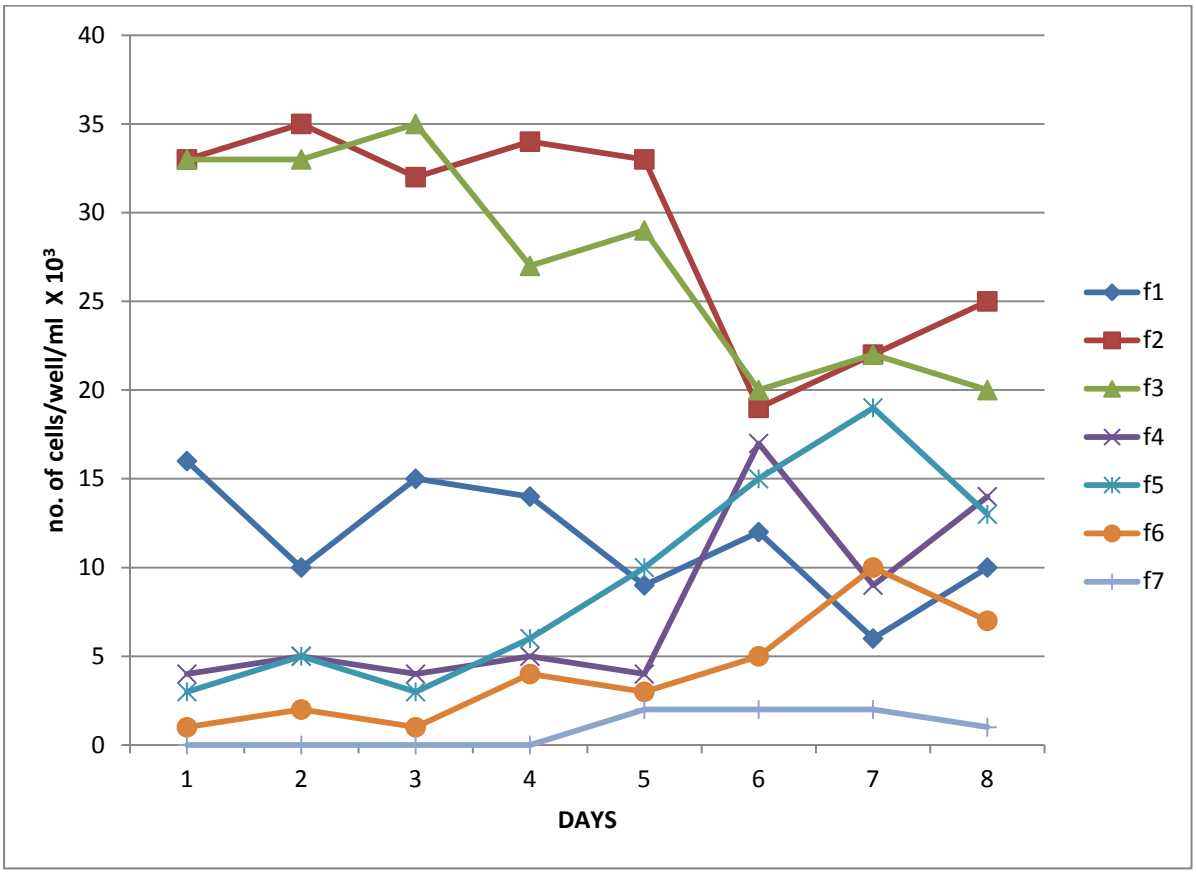


TABLE 35 – SUBPOPULATION PROPORTION OF 90 CELLS OF DECIDUOUS TOOTH SAMPLE 3 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	16	33	33	4	3	1	-
2	10	35	33	5	5	2	-
3	15	32	35	4	3	1	-
4	14	34	27	5	6	4	-
5	9	33	29	4	10	3	2
6	12	19	20	17	15	5	2
7	6	22	22	9	19	10	2
8	10	25	20	14	13	7	1

GRAPH 27 – SUBPOPULATION ANALYSIS OF DECIDUOUS TOOTH SAMPLE 6

f1 to f7 [KLAUS BAYREUTHER et al 1988]

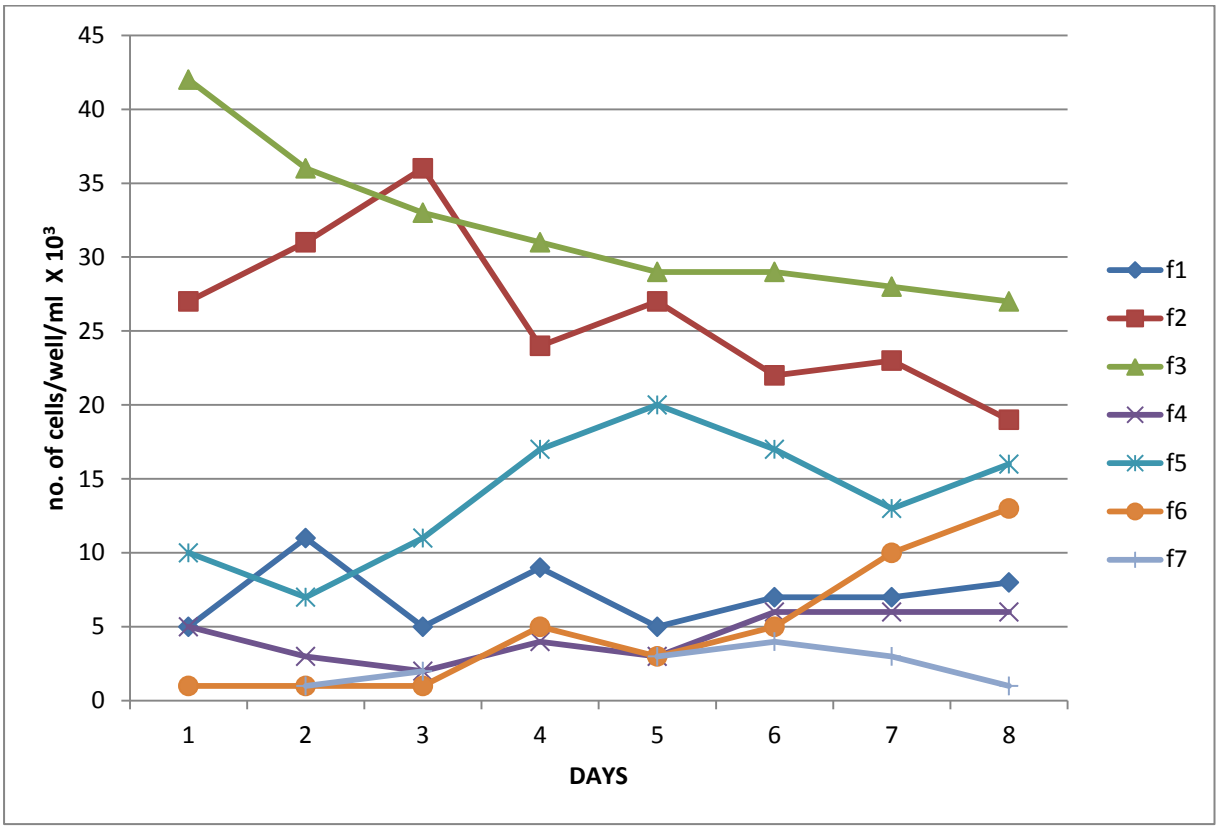


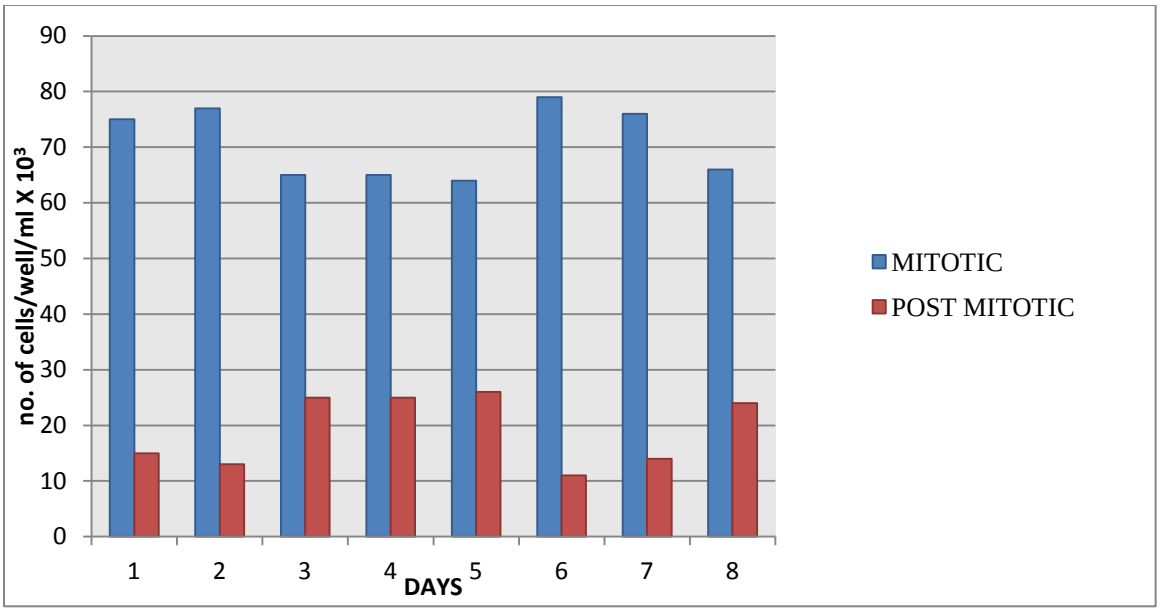
TABLE 36 – SUBPOPULATION PROPORTION OF 90 CELLS OF DECIDUOUS TOOTH SAMPLE 6 FOR 8 DAYS

DAYS	F1	F2	F3	F4	F5	F6	F7
1	5	27	42	5	10	1	-
2	11	31	36	3	7	1	1
3	5	36	33	2	11	1	2
4	9	24	31	4	17	5	-
5	5	27	29	3	20	3	3
6	7	22	29	6	17	5	4
7	7	23	28	6	13	10	3
8	8	19	27	6	16	13	1

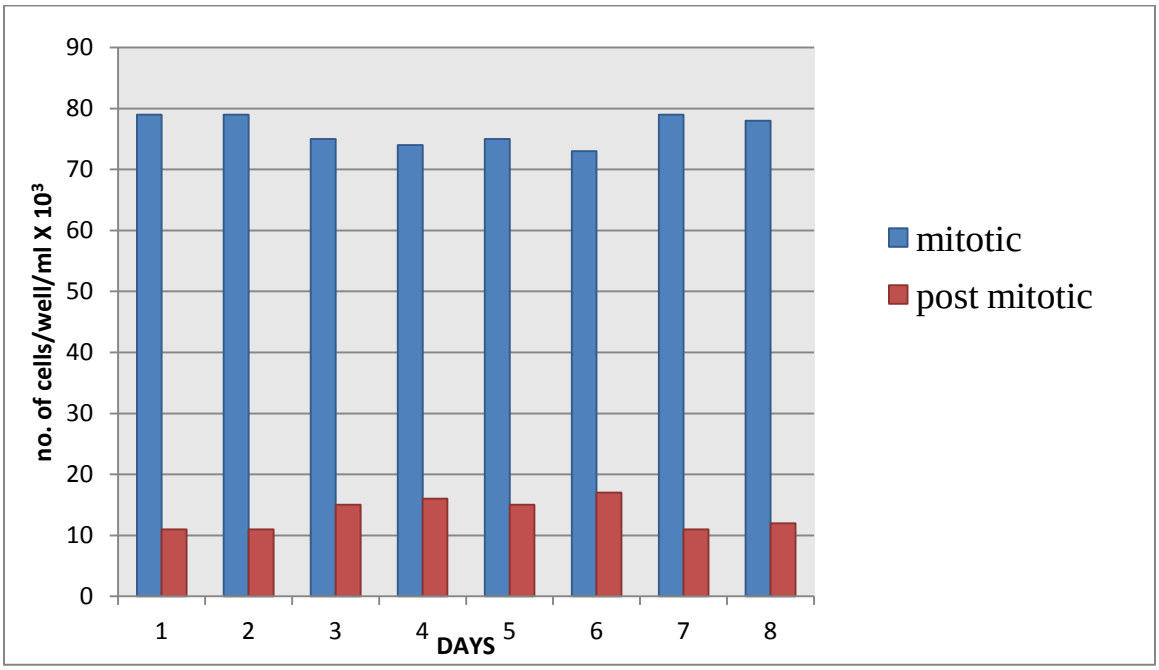
MITOTIC AND POST MITOTIC SUBTYPE ANALYSIS

Dental Pulp Stem cells – permanent tooth sample

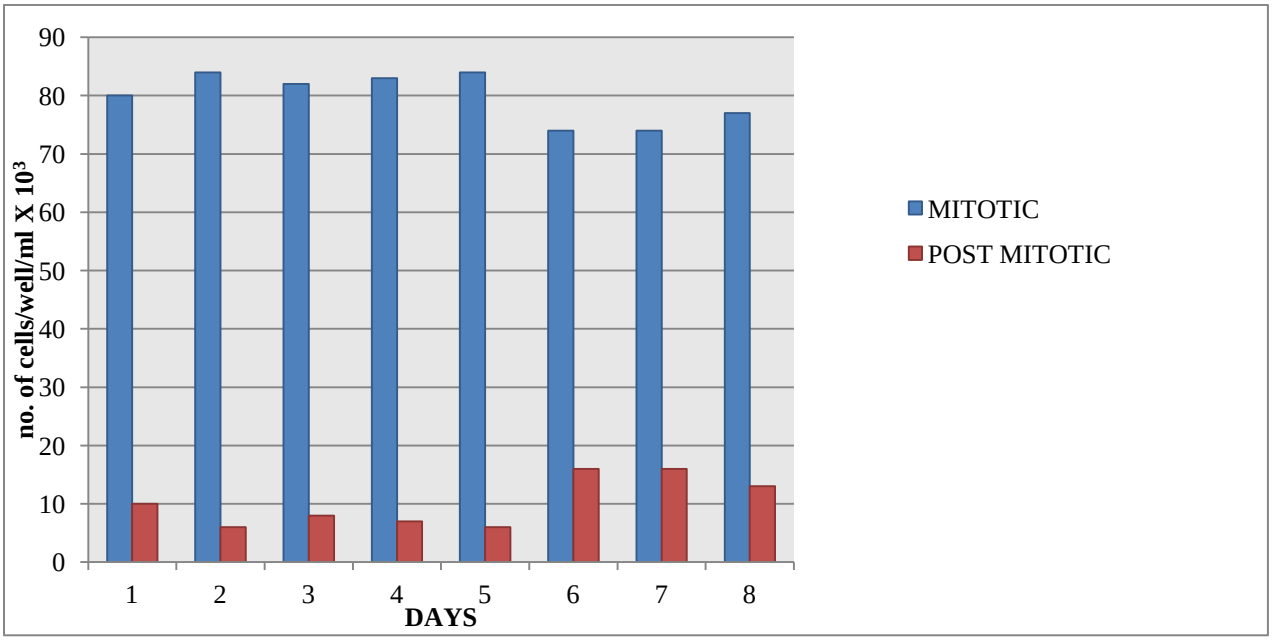
GRAPH 28: permanent sample 2



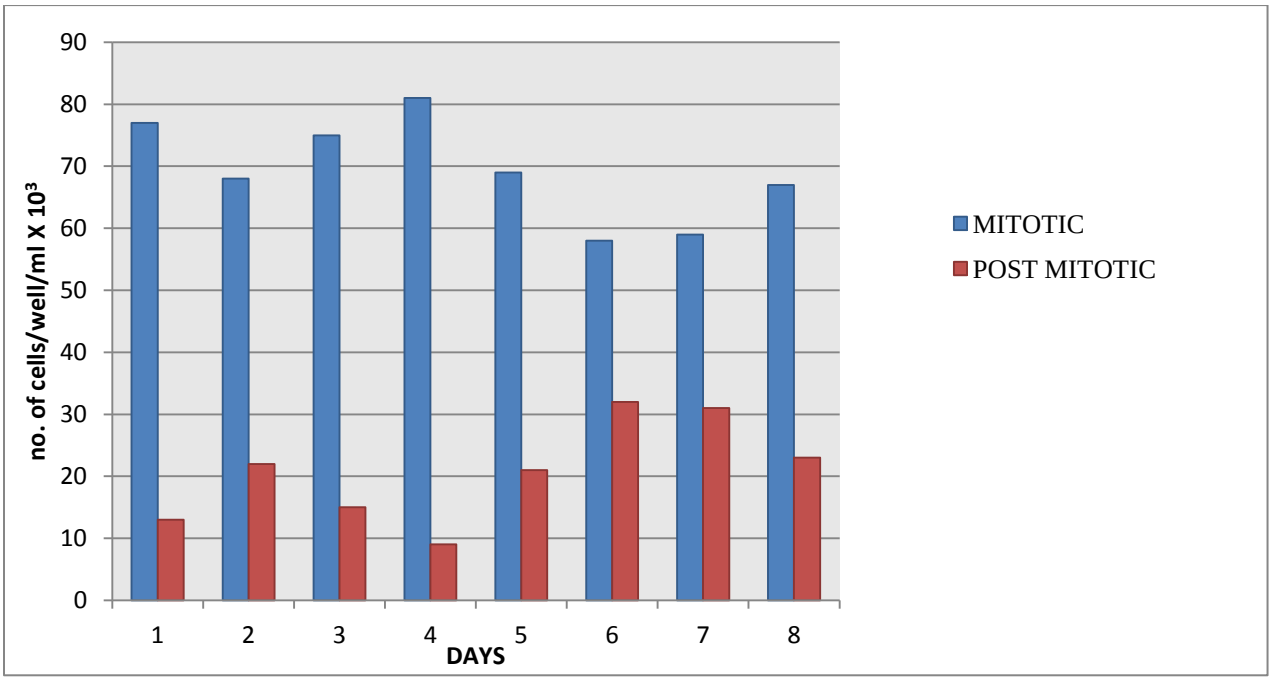
GRAPH 29: permanent sample 3



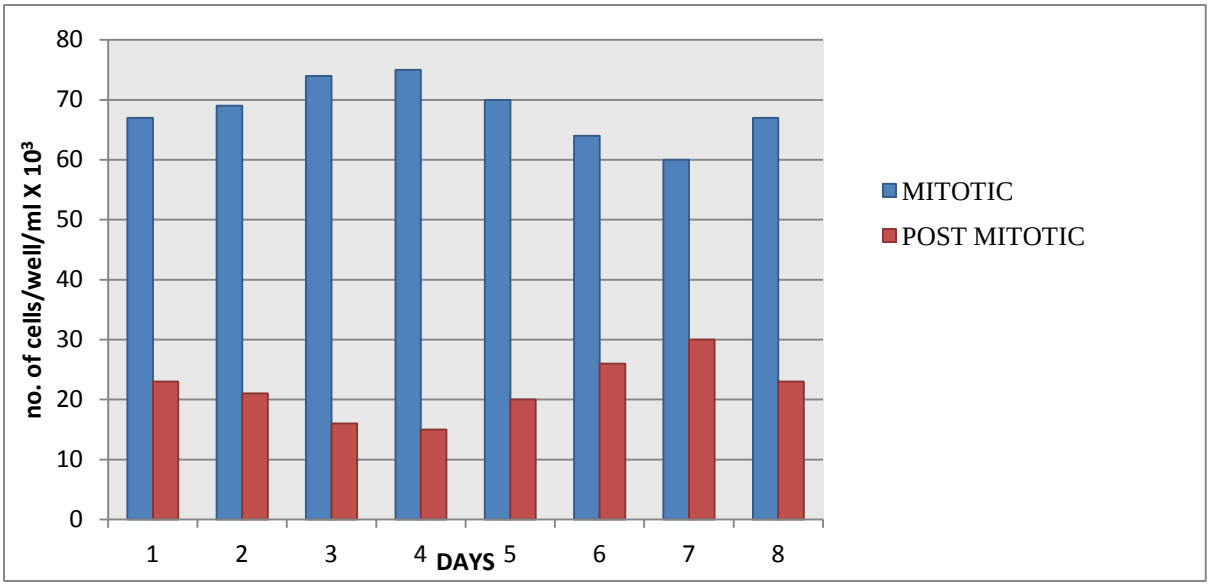
GRAPH 30: permanent sample 6



GRAPH 31: Permanent sample 9

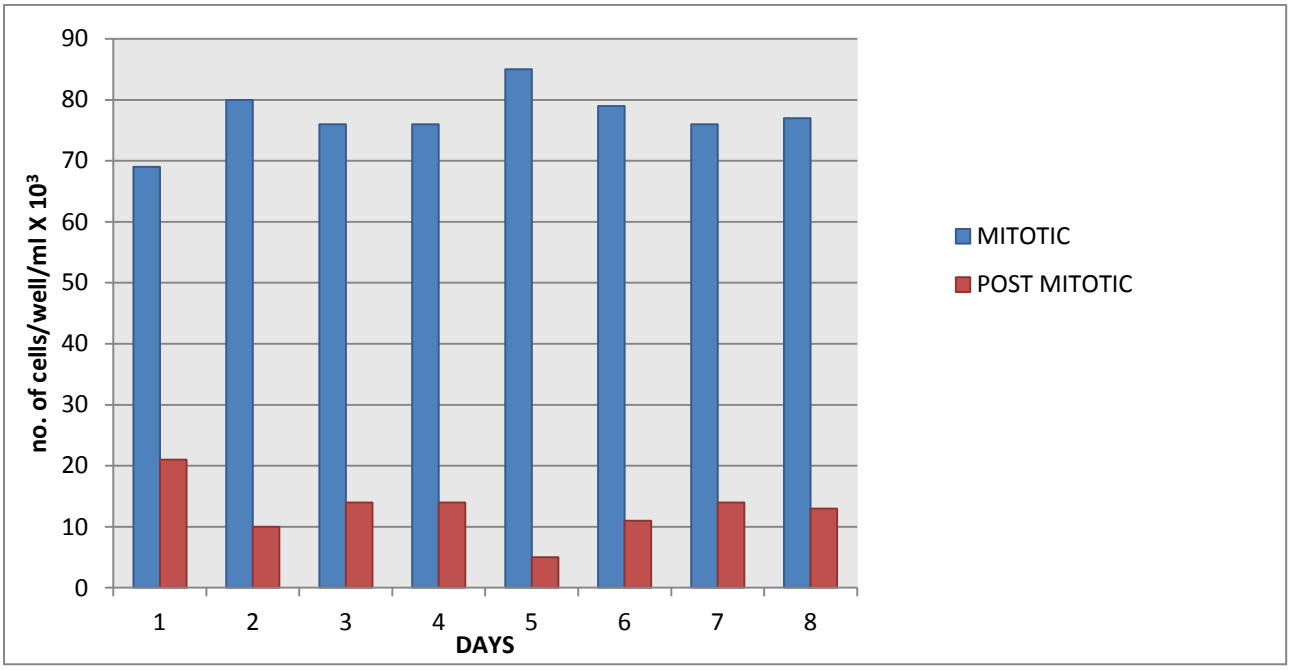


GRAPH 32: Permanent sample 12

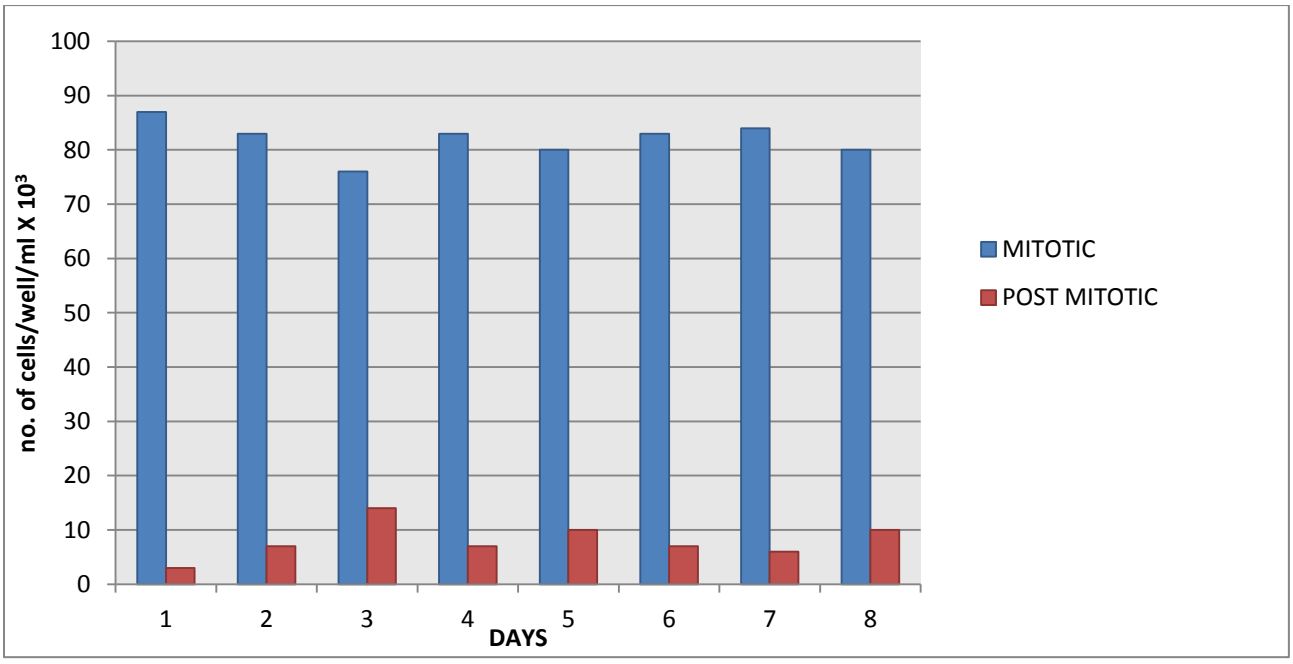


Stem Cells from Human Exfoliated Deciduous Teeth – Deciduous Teeth Samples

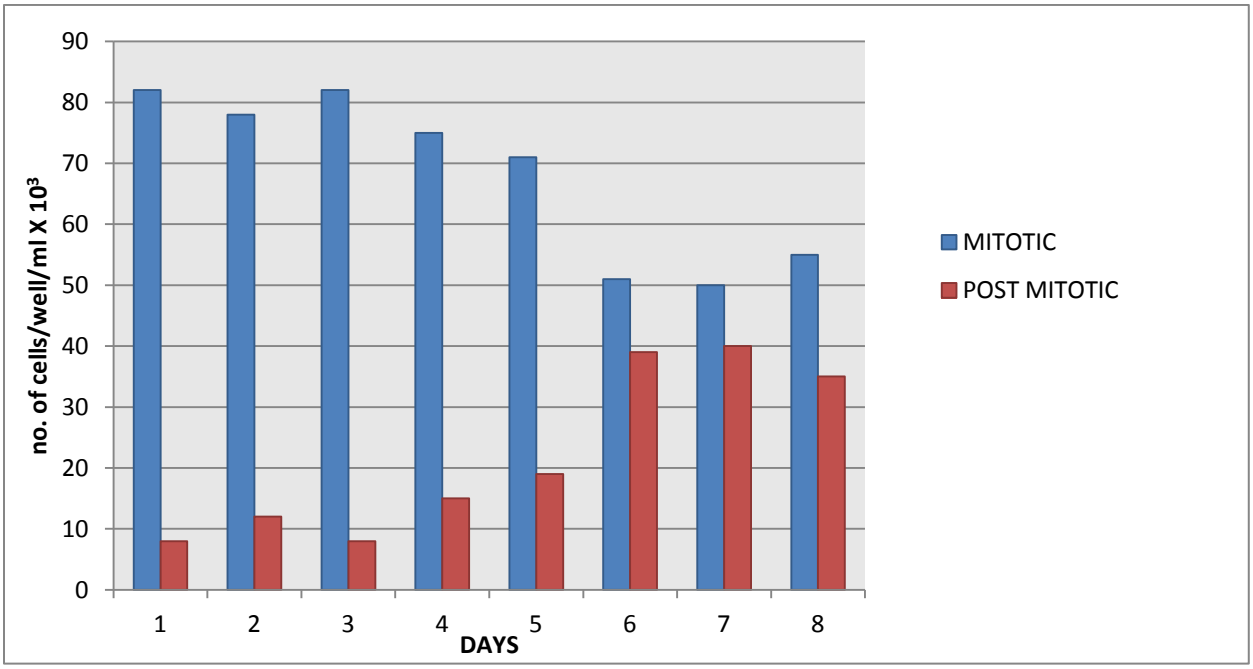
GRAPH 33: Deciduous sample 1



GRAPH 34: Deciduous sample 2



GRAPH 35: Deciduous sample 3



GRAPH 36: Deciduous sample 6

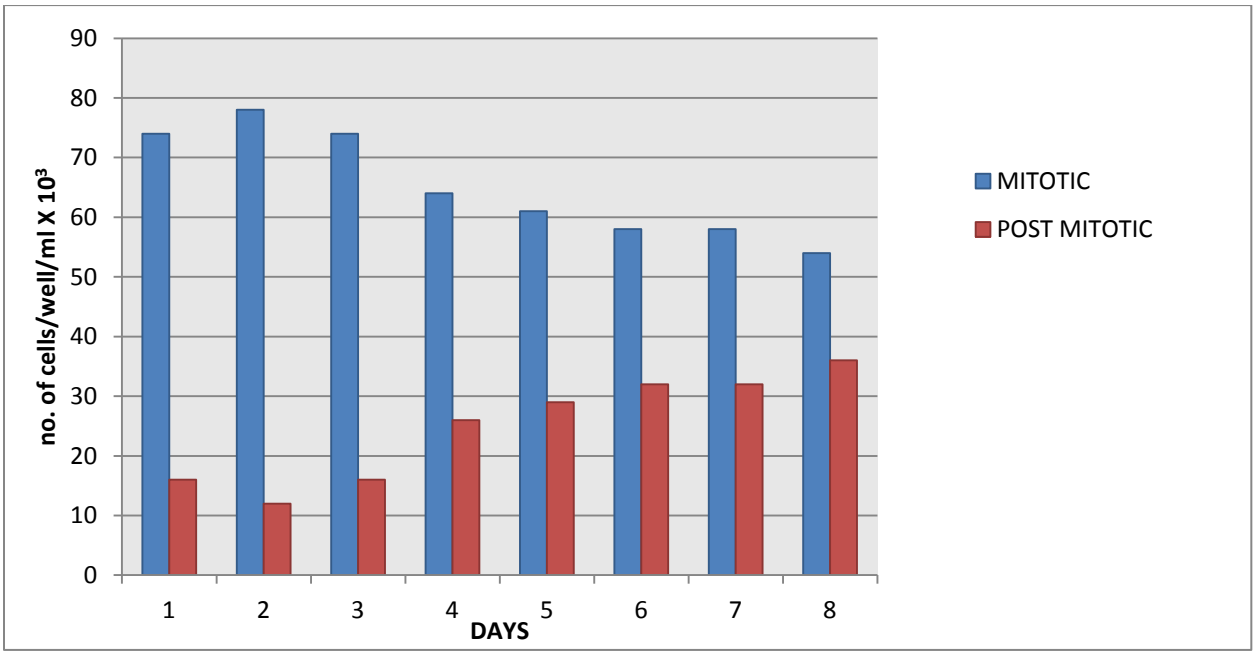


TABLE 37: Mitotic & Post mitotic Analysis of Dental Pulp Stem Cell

PERMANENT SAMPLES	CORRELATION COEFFICIENT	P-VALUE
Permanent sample 2	-1.00	.000*
Permanent sample 3	-1.00	.000*
Permanent sample 6	-1.00	.000*
Permanent sample 9	-1.00	.000*
Permanent sample 12	-1.00	.000*

*- Statistically significant at 5% level

TABLE 38: Mitotic & Post Mitotic Analysis of Stem Cells from Human Exfoliated Deciduous Teeth

DECIDUOUS SAMPLES	CORRELATION COEFFICIENT	P-VALUE
Deciduous sample 2	-1.00	.000*
Deciduous sample 3	-1.00	.000*
Deciduous sample 6	-1.00	.000*
Deciduous sample 9	-1.00	.000*
Deciduous sample 12	-1.00	.000*

*- Statistically significant at 5% level

TABLE 39: comparison of mitotic and post mitotic populations between the dental pulp Stem cells and stem cells from human exfoliated deciduous teeth

SAMPLE	P - Value
DPSC	.99
SHED	.99

TABLE 40: A Comparative Subpopulation Analysis between Dental Pulp Stem Cells & Stem Cells from Exfoliated Deciduous Teeth (MOLLENHAUER et al 1986)

		F1	F2	F3
Permanent	mean	38.67	36.96	34.00
Deciduous		33.78	35.92	39.63
p-value		.32	.83	.26

Seeding Efficiency In Percentage / Population Doubling Time In Hours

TABLE 41: Dental Pulp Stem Cells (DPSCs)

Sample	Seeding efficiency %	Population doubling time (hours)
Permanent Sample 2	112	43.92
Permanent Sample 3	101.83	111.99
Permanent Sample 6	57.41	144.62
Permanent Sample 9	37	111.32
Permanent Sample 12	63.90	32.43

TABLE 42: Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

SAMPLE	Seeding efficiency %	Population doubling time (hours)
Deciduous sample 1	119.42	246.79
Deciduous sample 2	42.59	13.51
Deciduous sample 3	83.33	68.18
Deciduous sample 6	43.5	73.87

TABLE 43: Comparison Between Dental Pulp Stem Cells & Stem Cells From Exfoliated Deciduous Teeth

Fibroblast subpopulation	P-value
Seeding Efficieny %	**1.000**
Population Doubling TIme	**.81**

Table 44 : Experimental Summary For Immunocytochemical Analysis Of STRO1 In DPSCs & SHED								
no:	**sample**	**fixation**	**PBS pH**	**primary dilution**	**primary incubation**	**secondary dilution**	**secondary incubation**	**result**
1	permanent sample 2	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
2	permanent sample 2	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
3	permanent sample 3	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
4	permanent sample 3	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
5	permanent sample 6	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
6	permanent sample 6	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
7	deciduous sample 1	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
8	deciduous sample 1	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
9	deciduous sample 2	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
10	deciduous sample 2	methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
11	deciduous sample 3	acetone	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
12	deciduous sample 3	acetone	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
13	permanent sample 9	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
14	permanent sample 9	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative

15	permanent sample 12	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
16	permanent sample 12	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
17	deciduous sample 1	Methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
18	deciduous sample 1	Methanol	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
19	deciduous sample 4	Acetone	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
20	deciduous sample 4	Acetone	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
21	deciduous sample 5	Acetone	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
22	deciduous sample 5	Acetone	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
23	deciduous sample 6	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative
24	deciduous sample 6	Para formaldehyde	7.26	1 -100	overnight	01:50	1 and 1/2 an hour	negative

Table 45 : Experimental Summary For Immunocytochemical Analysis Of CD 106/ Vcam In DPSCs & SHED										
sample no	**sample details**	**fixation**	**antigen retrival**	**primary dilution**	**primary incubation**	**secondary dilution**	**secondary incubation**	**PBS**	**blocking**	**result**
1	permanent sample 2	methanol	------	1-100	1 hour	1 -100	1 hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative
2	deciduous sample 2	methanol	------	1-100	1 hour	1 -100	1 hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative
3	permanent sample 2	methanol	------	1-100	1 hour	1 -100	1 hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative
4	deciduous sample 2	methanol	------	1-100	1 hour	1 -100	1 hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative
5	permanent sample	methanol	------	01:50	overnight	1 -100	1 hour	ice cold PBS pH - 7.13	1% BSA in PBST for 30 minutes	negative
6	permanent sample 2	methanol	------	1 -100	overnight	1 -100	1 and half an hour	ice cold PBS pH - 7.13	1% BSA in PBST for 30 minutes	negative
7	permanent sample 2	methanol	------	01:50	overnight	1 -100	1 hour	ice cold PBS pH - 7.13	1% BSA in PBST for 30 minutes	negative
8	permanent sample 2	methanol	------	1 - 100	overnight	1 -100	1 and half an hour	ice cold PBS pH - 7.13	1% BSA in PBST for 30 minutes	negative
9	deciduous sample 6	methanol	------	01:50	1 hour	1 - 100	1 hour	ice cold PBS pH - 7.14	1% BSA in PBST for 30 minutes	negative

10	Deciduous sample 1	methanol	------	01:50	overnight	1 - 100	1hour	ice cold PBS pH - 7.14	1% BSA in PBST for 30 minutes	negative
11	deciduous sample 4	acetone	------	01:50	1 hour	1- 100	1 hour	ice cold PBS pH - 7.14	1% BSA in PBST for 30 minutes	negative
12	deciduous sample 4	acetone	------	01:50	overnight	1-100	1 hour	ice cold PBS pH - 7.14	1% BSA in PBST for 30 minutes	negative
13	Permanent sample 6	paraform aldehyde	------	01:50	1 hour	1-100	1 hour	ice cold PBS pH - 7.14	1% BSA in PBST for 30 minutes	negative
14	permanent 6	paraform aldehyde	------	01:50	overnight	1-100	1 hour	ice cold PBS pH - 7.14	1% BSA in PBST for 30 minutes	negative
15	Deciduous sample 1	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:25	1 hour	01:50	1 hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative
16	Deciduous sample 1	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:25	overnight	01:50	1 hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative
17	deciduous sample 3	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:50	1 hour	01:50	1 hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative
18	deciduous sample 1	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:50	overnight	01:50	1hour	ice cold PBS pH - 7.23	1% BSA in PBST for 30 minutes	negative

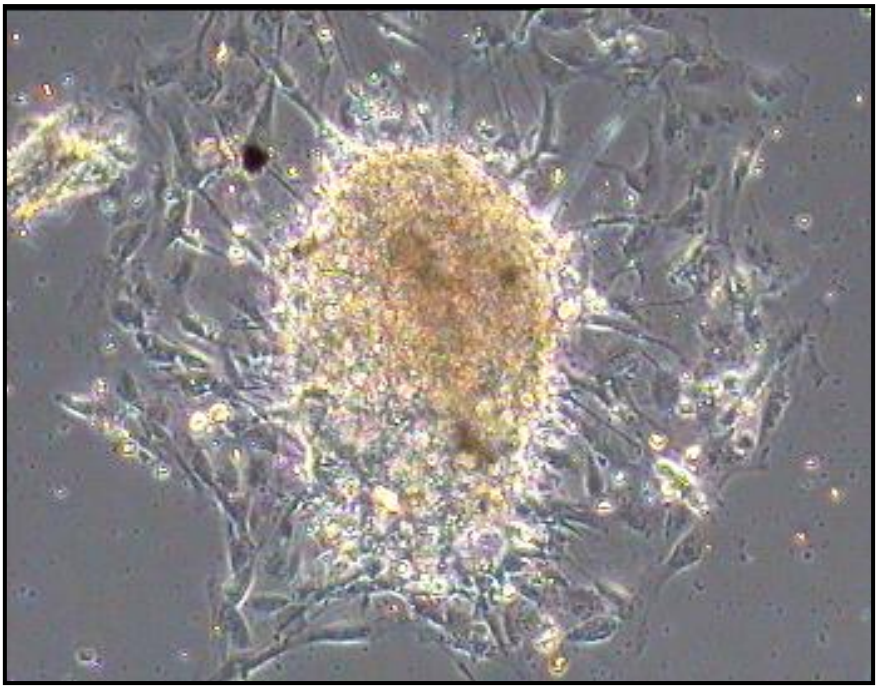
19	permanent sample 2	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:10	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
20	deciduous sample 2	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:20	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
21	deciduous sample 2	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:10	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
22	Permanent sample 2	methanol	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:20	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
23	permanent sample 2	methanol	------	01:10	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
24	deciduous sample 2	methanol	------	01:20	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
25	deciduous sample 2	methanol	------	01:10	overnight	01:25	1 hour	PBS pH - 7.42	------	Negative
26	permanent sample 2	methanol	------	01:20	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
27	deciduous sample 3	acetone	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:10	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative

28	Deciduous sample 3	acetone	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:20	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
29	Deciduous sample 3	acetone	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:10	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
30	deciduous –sample 3	acetone	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:20	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
31	deciduous sample 3	acetone	------	01:10	1 hour	01:25	1 hour	PBS pH - 7.42	------	Negative
32	deciduous sample 3	acetone	------	01:20	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
33	deciduous sample 3	acetone	------	01:10	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
34	deciduous – sample 3	acetone	------	01:20	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
35	deciduous sample 6	paraform aldehyde	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:10	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
36	deciduous sample 4	paraform aldehyde	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:20	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative

37	deciduous sample 4	paraform aldehyde	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:10	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
38	deciduous sample 6	paraform aldehyde	TRIS buffer 9.5 pH at 95°c for 10 minutes	01:20	overnight	01:25	1 hour	PBS pH - 7.42	------	Negative
39	deciduous sample 6	paraform aldehyde	------	01:10	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
40	deciduous sample 4	paraform aldehyde	------	01:20	1 hour	01:25	1 hour	PBS pH - 7.42	------	negative
41	deciduous sample 3	paraform aldehyde	------	01:10	overnight	01:25	1 hour	PBS pH - 7.42	------	negative
42	Deciduous sample 6	paraform aldehyde	------	01:20	overnight	01:25	1 hour	PBS pH - 7.42	------	negative

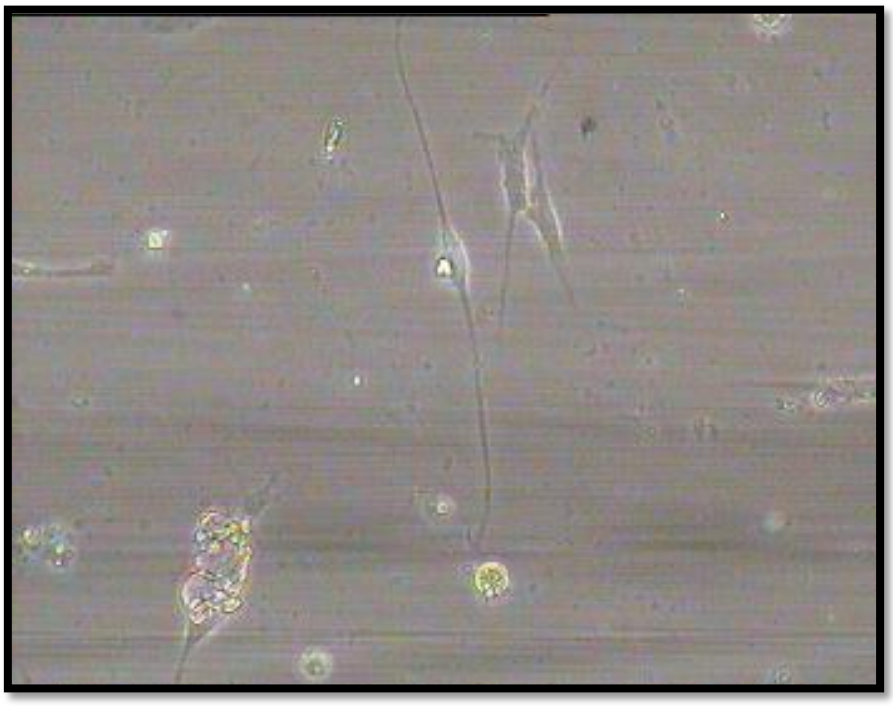
Photographs

FIGURE 1



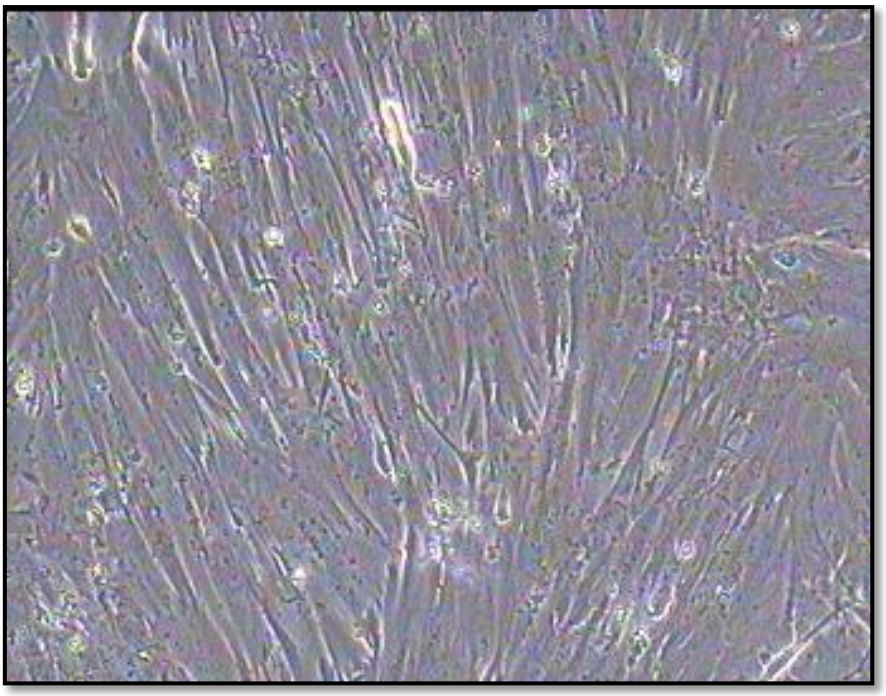
Cell emerging from a disaggregated tissue (20 X)

FIGURE 2



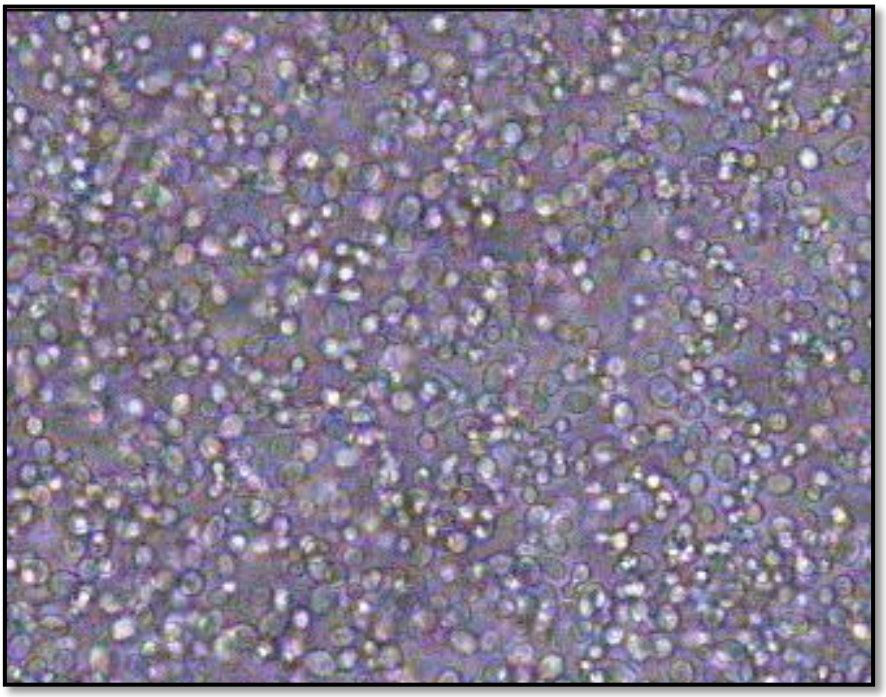
Individual cells seen 48 hours after plating (20 X)

FIGURE 3



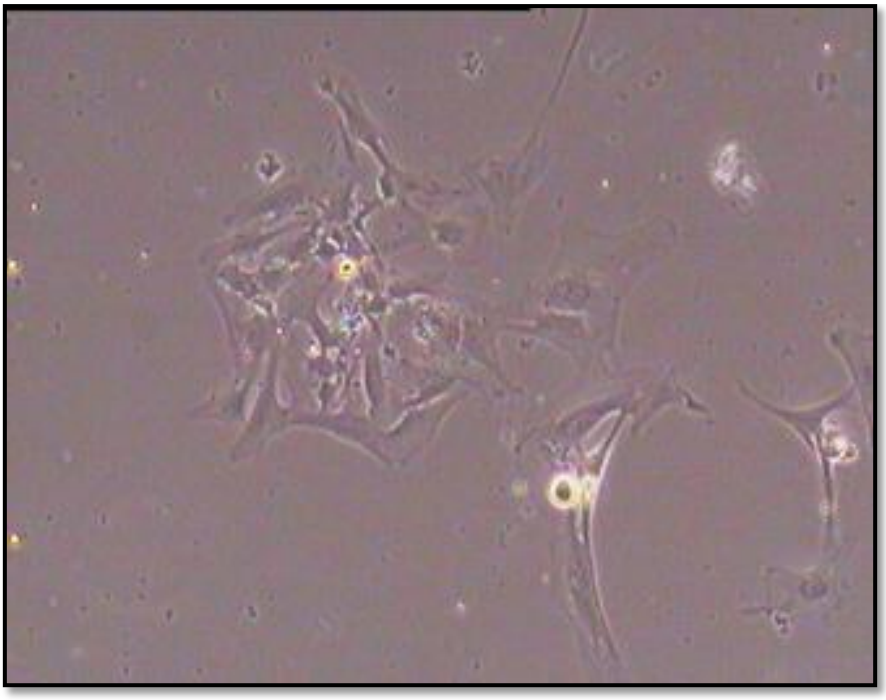
Confluent culture (20 X)

FIGURE 4



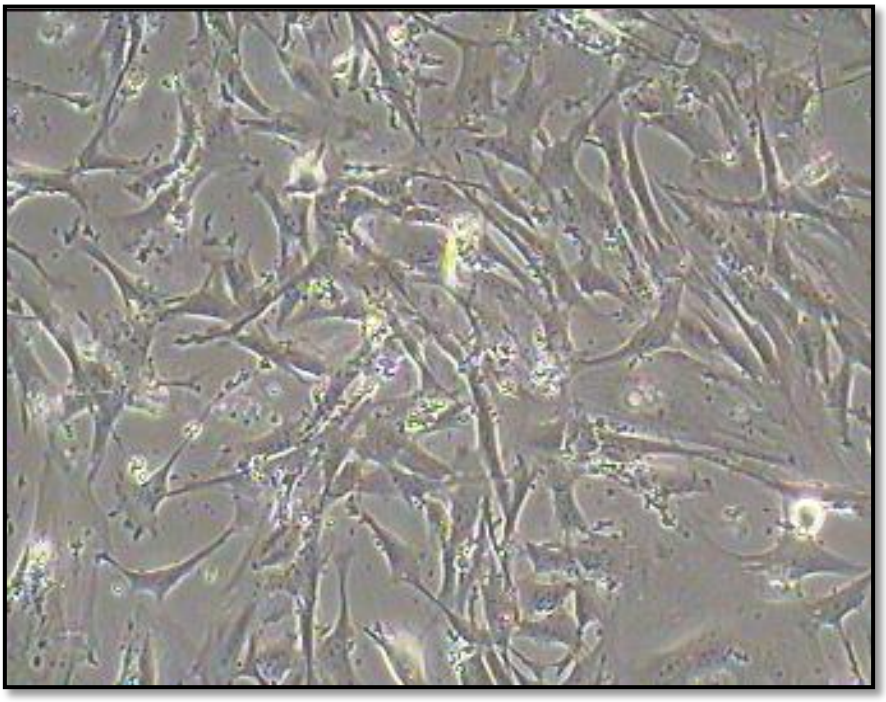
Budding structures suggestive of fungi (20 X)

FIGURE 5



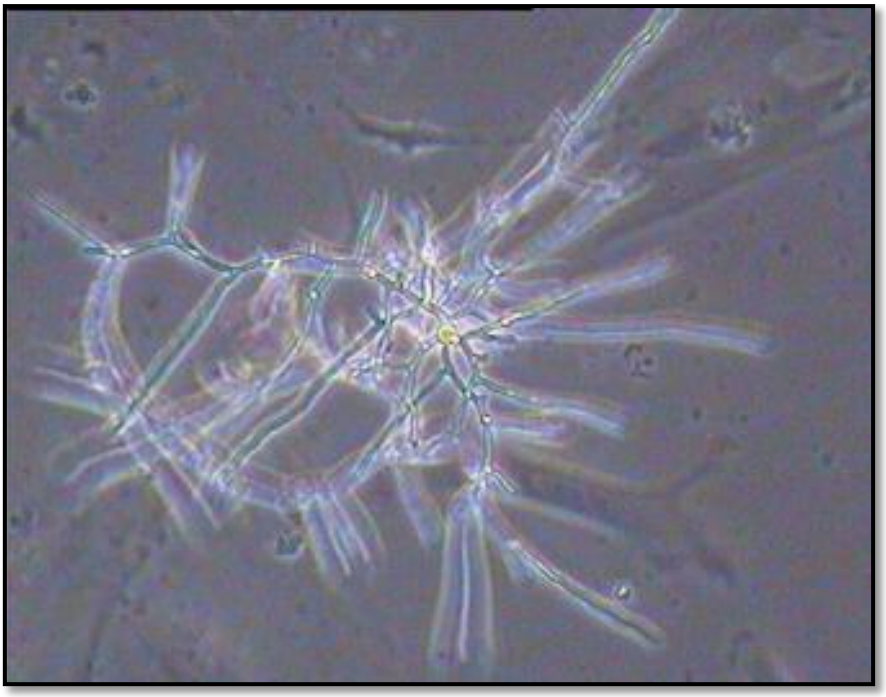
Small colony of cells(10 X)

FIGURE 6



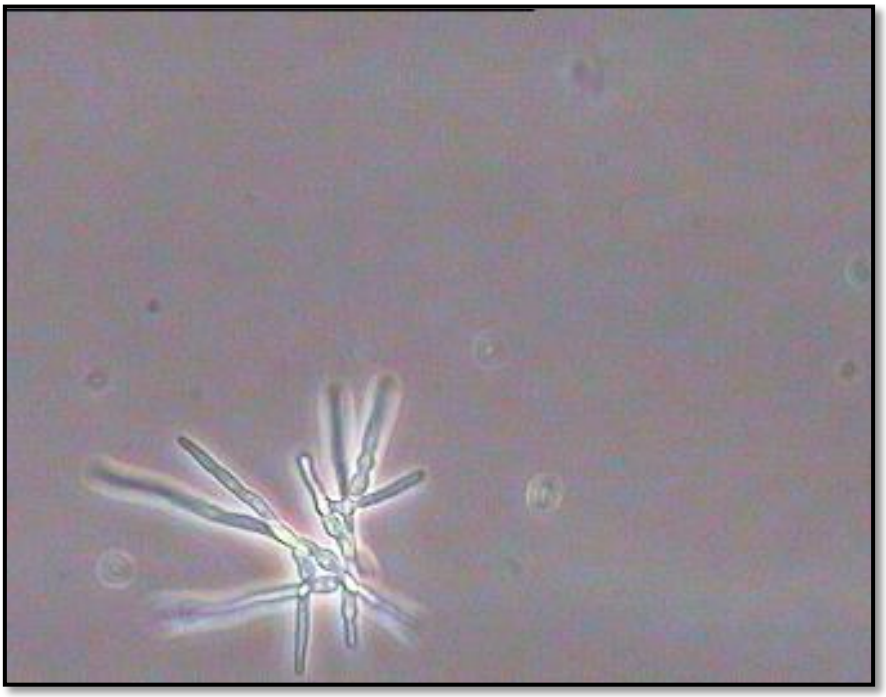
Sub confluent culture (20 X)

FIGURE 7



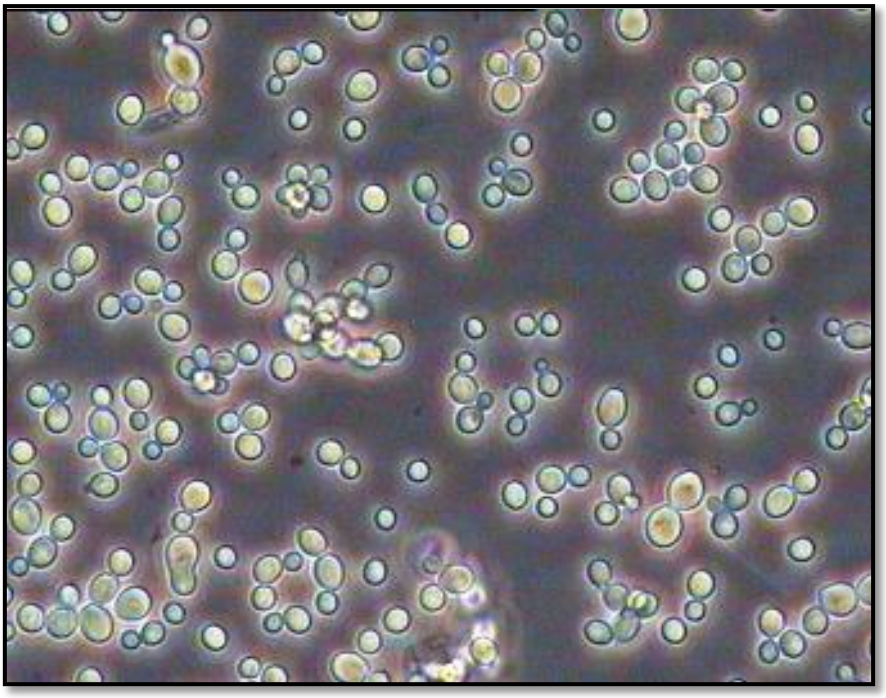
Branched filamentous hyphae (20 X)

FIGURE 8



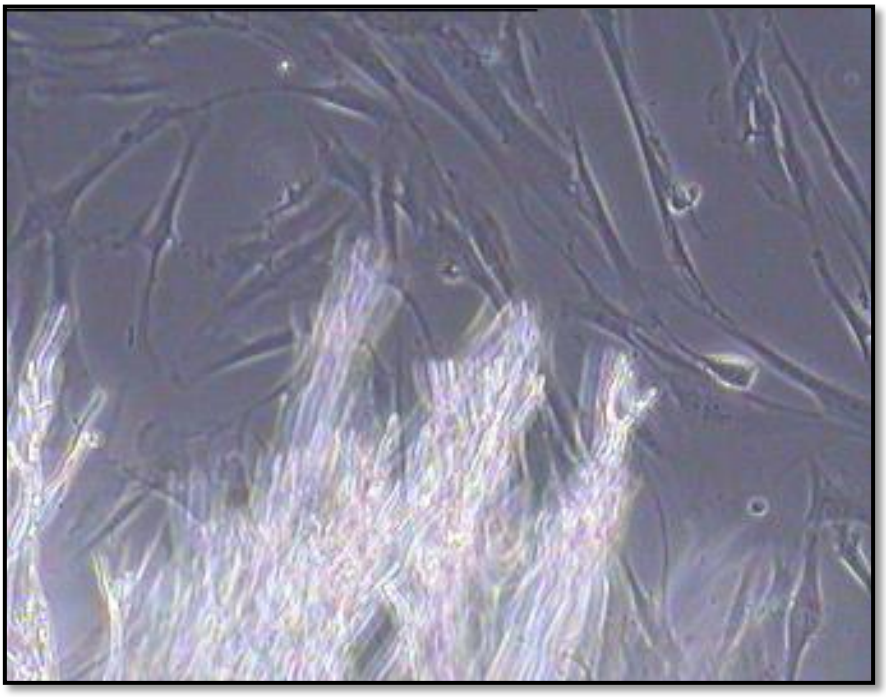
Segmented hyphae (20 X)

FIGURE 9



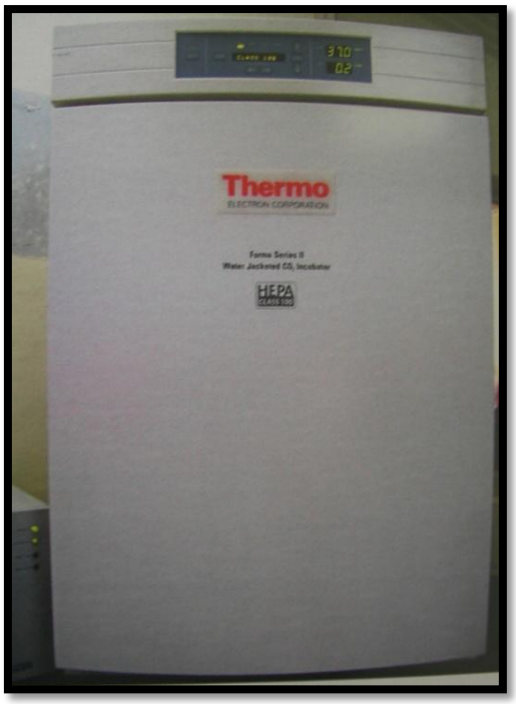
Bud like structures (20 X)

FIGURE 10



Filamentous Structure Suggestive of Fungi

FIGURE 11

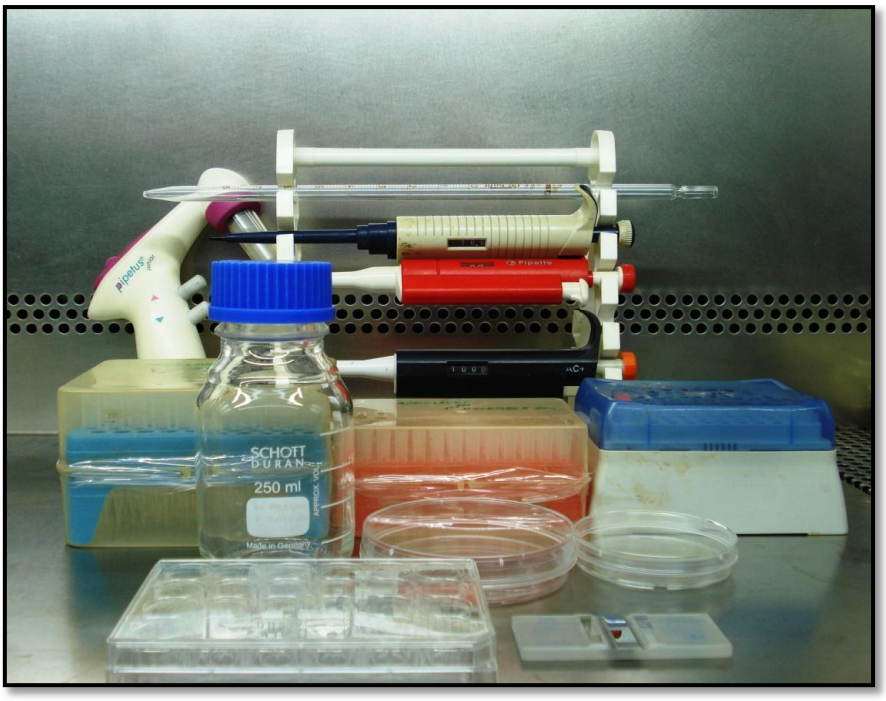


CO_2 Incubator

FIGURE 12

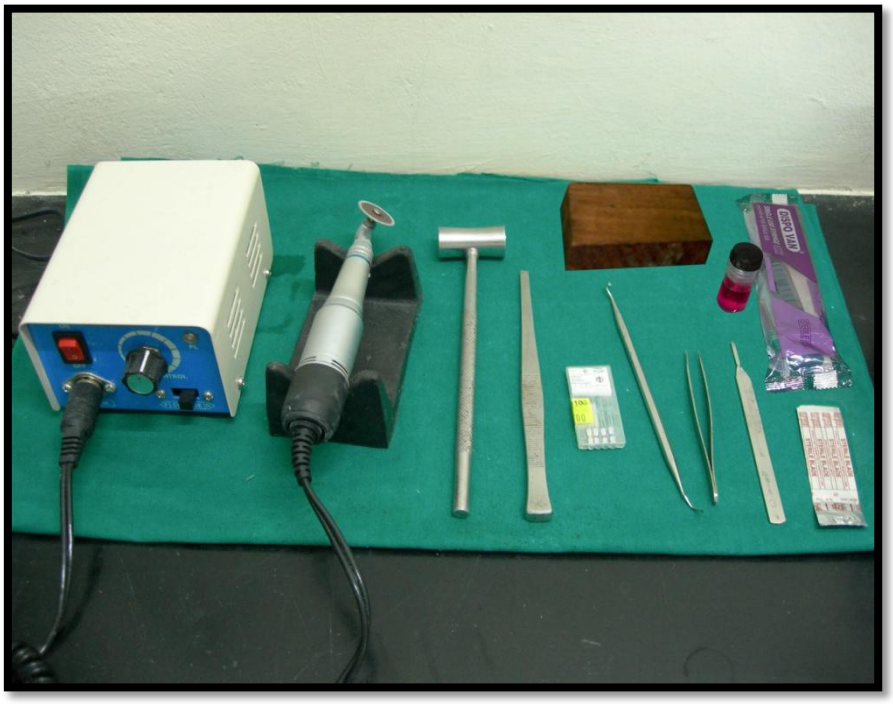
Laminar Flow

FIGURE 13



Cell Culture Armamentarium

FIGURE 14



Pulp Tissue Isolation Armamentarium

FIGURE 15

Cell Culture Reagents

FIGURE 16

Immunocytochemistry Reagents

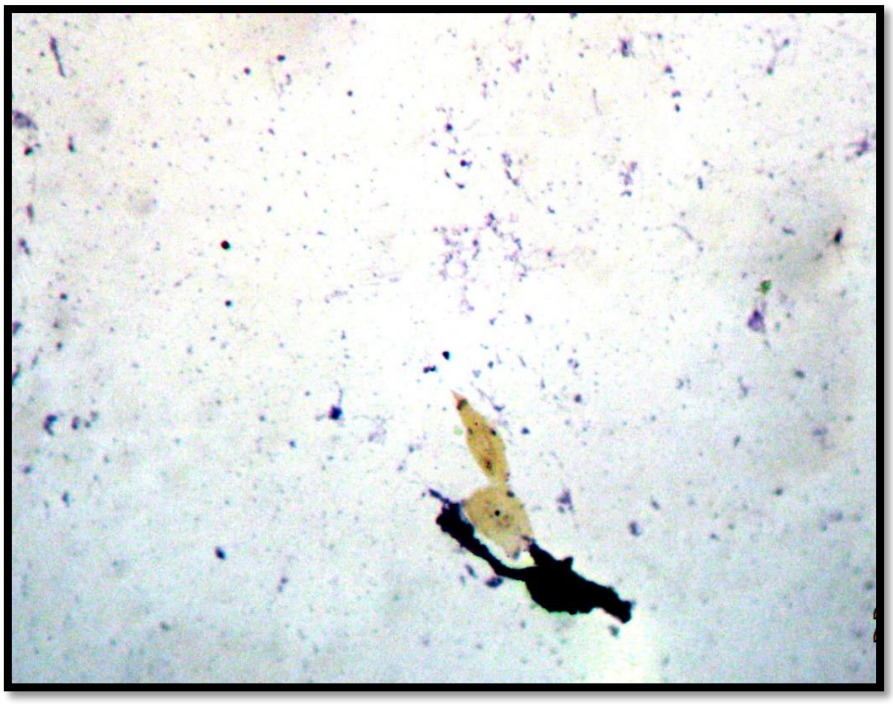
FIGURE 17

Primary and Secondary Antibodies

FIGURE 18

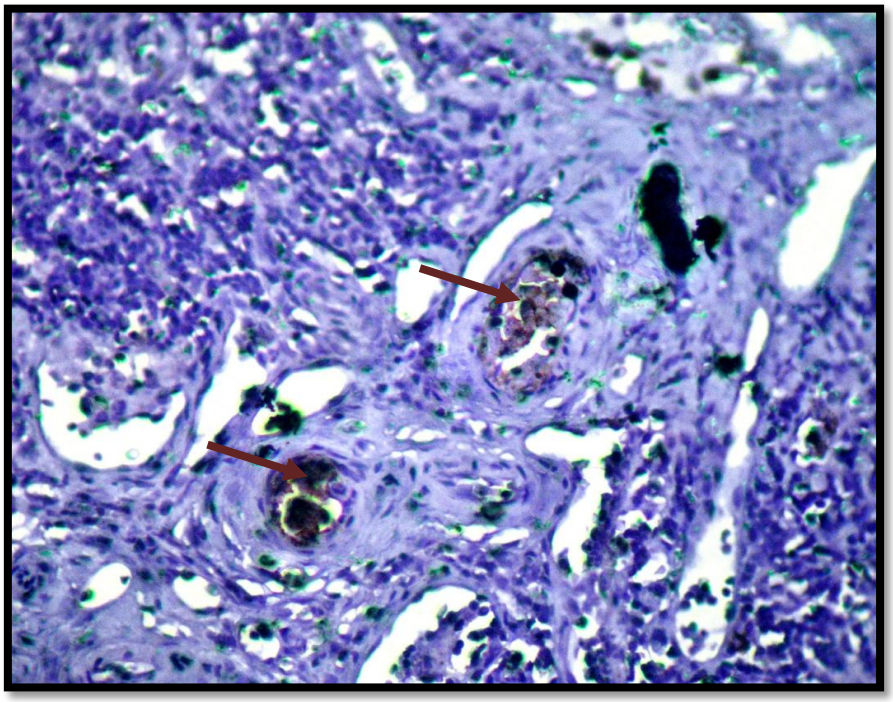
Cryo boxes

FIGURE 19



Expression of STRO 1 in osteo sarcoma cells (positive control)

FIGURE 20



Expression of CD 106 in lymphnodes (positive control) arrows indicate staining

Discussion

The chance of utilising stem cells in clinical dentistry opens completely new approaches in therapeutics. Of the various stem cells considered, mesenchymal stem cells are a better option as they have the multipotential differentiation. Though bone marrow mesenchymal stem cells have been studied extensively, dental pulp represents a source of stem cells, that is easily available, as the impacted third molars, permanent tooth extracted for orthodontic reasons and exfoliated deciduous teeth are accessible sources of stem cells.

In this study, we attempted to compare the characteristics of Dental Pulp Stem cells(DPSC) and Stem cells from Human Exfoliated Deciduous teeth(SHED) and their expression of cell surface markers STRO1 and CD 106 or Vascular Cell Adhesion Molecule.

The isolation protocol of this study was similar to that as described by **Gronthos et al 2000 [9], Miura et al 2003 [1], Shi and Gronthos [2] 2003** and as described in **Ian Freshney's Textbook of human cell culture [32]** for isolation of dental pulp stem cells. We transferred the tooth specimen in the media alpha modification of minimal essential medium containing the double strength of antibiotics but without the serum. The specimen was transported to the cell culture laboratory by maintaining the temperature at 4°C. On reaching the laboratory, the specimen was completely rinsed in Phosphate buffered saline to remove blood clot or debris that might contaminate the pulp tissue while extirpation. The tooth was split at the cementoenamel junction; the pulp tissue thus extirpated from the pulp chamber was put into leak proof screw cap vial containing 2 ml of the medium containing antibiotics. The pulp tissue was subjected to enzymatic disaggregation after which the tissue was suspended in media in the tissue culture

plate. The media was changed after 48 hours and every 3rd day till confluency was reached and then the plates were subcultured. Cells from the third to fourth passage were utilised for growth characteristics and phenotypic analysis.

We successfully isolated 5 DPSC from 12 samples of permanent teeth and 6 SHED from 9 deciduous teeth. Inspite of the aseptic precautions we undertook during the isolation procedure some of our samples permanent sample 1 , 4,5, 7, 8 , 10 and 11 deciduous sample 7, 8 and 9) succumbed to contamination. The risk of contamination was increased when there was a delay in the transfer of the tooth specimen immediately into the media after the surgery. During the pulp extirpation, we observed that pulp from deciduous teeth was lesser in quantity compared to that of permanent teeth. Hence we undertook only 5 to 6 hours of enzymatic disaggregation of the deciduous pulp compared to that of 16 to 18 hours of enzymatic disaggragation of the permanent pulp.

For the growth characteristics analysis, the cells from the third passage were plated in 24 well plate at the rate of 12,000 cells /well/ ml. The cell count was then calculated daily by random selection of the wells for 8 consecutive days and finally the growth curve was plotted.

Seeding efficiency is the percentage of the inoculums that attached to the substrate after 12 hours or the first day count. It implies only the cell viability or survival but not the proliferation capacity. In our study the seeding efficiency of permanent samples ranged from 37 to 112 while that of deciduous samples ranged from 42.59 to 119.42. The difference in the seeding efficiency could be probably attributed to the fact that DPSCs had better viability than the SHEDs. Though

there was a difference in the seeding efficiency between all samples and also between the dental pulp stem cells from permanent and deciduous teeth, this difference was not statistically significant.

Population doubling time is the interval period required by a cell population to double at the middle of the logarithmic phase of growth. In our study, the population doubling time of dental pulp stem cells ranged from 32.43 to 144. 62 hours while that of stem cell from deciduous teeth ranged from 13.51 to 246.79 hours.

Though in our study the seeding efficiency and the population doubling time was not similar in all the samples and also between the stem cell from dental pulp and deciduous teeth there was no statistical significance**.**

Cells from the third to fourth passage were plated in three different plates at a density of 5000 cells per plate and were subjected to subpopulation analysis. Based on the morphology of cells as described by **Mollenhauer et al 1986** [26], the cell were observed daily for 8 consecutive days and they characterised the cells as F1 – spindle shaped cells with higher proliferation potential , F2 – epitheloid cells with less proliferation and F3 – stellate with least proliferation ability.

Our subpopulation analysis showed that though there were few F1 phenotypes that had faster proliferation rate, the major proportion of the cells we observed were F2 and F3 that had lesser proliferation rate compared to that of F1. These F2 and F3 cells were differentiating cells with less proliferation. Both DPSCs and SHEDs isolated and cultured in our study had longer doubling time.

We also analyzed the cells based on the description by **Klaus Bayreuther et al 1988** [27] .[Mitotic fibroblast subtypes F1 – small spindle shaped cells ,F2 – small epitheloid cells and F3 – larger pleomorphic epitheloid cells Postmitotic subtypes F4 – large spindle shaped cells ,F5- larger epitheloid cells, F6- largest epitheloid cells and F7-degenerating fibroblasts]. Based on our data, we found that there was difference between all the samples and also between the stem cell from permanent and deciduous teeth though there was no statistically significant difference between the two populations.

Antibodies and kits from different molecular laboratories, immunostaining, western blot and Enzyme Linked Immuno Sorbent Assay (ELISA) assays are the most important techniques available for cell line categorization. Antibody localization is assessed either by fluorescence, in which the antibody is conjugated to a fluorochrome, such as fluorescein or rhodamine, or by assessing precipitated product deposited from the activity of horseradish peroxidase or alkaline phosphatase conjugated to the antibody. [30]

An assorted panel of antibodies includes embryonal markers like the Oct-4 , Nanog and SSEA . Bmi , Notch and Sonic hedgehog also extends the list by playing a role in controlling the self renewal and differentiation properties of stem cells. Haematopoietic stem cell markers includes CD 34, CD133, ABCG2, Sca-1 . Nestin, Neurotrophin R (NTR) and PSA-NCAM adds to the list of neural stem cell markers Mesenchymal/Stromal Stem Cell Markers includes STRO1, VCAM (CD106), CD73, or lymphocyte-vascular adhesion protein2. Runx 2 acts early to promote osteoblastic differentiation. Sox9 to promote chondrocytic

differentiation. Over expression of Sox5, Sox 6 and Sox 9 in cultured cells and ectopic expression of Sox9 in mice induce the expression of type II collagen.

A systematic review on cell surface characterization of adult mesenchymal stem cells by **Mafi P, Hindocha S, Mafi R et al 2011** [31] was done. They concluded that the surface markers for mesenchymal stem cells like CD105, CD90, CD44, CD73, CD29, CD13, CD34, CD146, CD106, CD54 and CD166 had a positive expression whereas the antibodies such as CD14, CD11b, CD49d, CD34, CD106, CD10 and CD31 had negative expression. It was also observed that in all the studies considered for this review, markers like CD10, CD34, CD45 and CD106 did not have a uniform expression in the cell types and it varied among the different studies. The variability of the expression of these markers can be attributed to the heterogenicity of the cell types, or to the different cell passages that were used to assess the expression of markers.

Mafi et al 2011[31,] stated that on the cell surface characterization, mesenchymal stem cells CD 106 or the Vascular cell adhesion molecule had varied expression. **Gronthos in 2000** also concluded that VCAM was very weakly expressed by DPSCs.

In a study by **S.Shi et al 2003** [2] for identification of locality of the stem cells niche, several markers of smooth muscle cells endothelial cells and pericytes were used. They concluded that the stem cell population of both bone marrow and dental pulp were present in the perivascular niches. But in our study the morphologically characterizable stem cells from the dental pulp of both permanent and deciduous teeth did not express the perivascular marker CD106 or vascular

cell adhesion molecule, though we found that the high endothelial venules of lymphnodes and macrophage like cells in the tissue sections expressed CD 106 (positive control as stated by ABCAM in the data sheet ab7219). This non-expression of the vascular marker can probably be attributed to the fact that the stem cells have various niches, probably the stem cells we isolated and cultured had a different niche apart from the perivascular niches. Heterogeneity of stem cells populations can also be considered as an attributable cause for the non-expression of the CD106.Further characterization is necessary to locate the niches of these pulp stem cells.

STRO1 was first observed in bone marrow stromal precursors. According to a study by **Adhikari S et al** [34] done on Osteosarcoma cell lines , the tumor initiating cells of the Osteosarcoma are also known to express STRO1. In the study to localize the cancer stem cell line of Osteosarcoma by **V A Siclari and L Qin 2010** [42], the Osteosarcoma cells lines which were used as positive control expressed STRO1. In our study, we observed the positive expression of STRO1 in Osteosarcoma cell lines but not in the cell populations which we isolated and cultured.

Ultimately the population of cells we isolated were STRO1 and CD 106 or vascular cell adhesion molecule negative. This could probably be attributed to heterogenous nature of the stem cells. This supports the requirement of panel of markers needed for further chacterization

In our subpopulation analysis, we also found more of differentiating population (F2 and F3) and this is supported by the study of **Jakub suchanek et al 2009** [17] who stated that the proportion of Fetal bovine serum (15 %) added to the media for the culture of the cells can be reduced which in turn reduces the chance of the differentiation of the cell population isolated.

Summary & Conclusion

- In this study we successfully isolated and cultured five Dental Pulp Stem Cells (DPSC) from twelve samples of permanent teeth and six Stem cells from Human Exfoliated Deciduous teeth (SHED) from nine deciduous teeth. We compared the growth characteristics and the phenotype of both the cell populations. We also evaluated the immuno histochemical expression of STRO1 and CD 106 on these cells.
- Estimation of growth curve and its derivatives revealed that the doubling time of DPSCs ranged from 37 hours to 144 hours. This long population doubling time indicate their loss of proliferation and entering the process of differentiation. SHED also had similar doubling time ranging from 13 hours to 246 hours
- The subpopulation analysis of both DPSCs and SHED revealed no statistically significant difference between the two groups. ($p>0.05$) .
- The seeding efficiency of DPSCs ranged from 37 to 112% but that of SHED ranged from 42.6 to 119.4.
- Both DPSCs and SHED had higher proportion of F2 subpopulation , indicating slow proliferation.
- The morphologically characterized DPSCs and SHED did not express the early mesenchymal markers, like STRO1 and VCAM.
- Further study using a larger panel of antibodies is required to better localize and characterizethe stem cell niches of DPSCs and SHED apart from their perivascular locations.
- Given the easy accessibility of pulp stem cell the molecular studies of DPSC and SHED with more number of samples should be done.

- In future such characterization would improve the scope and utility of these cells in clinical dentistry and therapeutics.

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Annexure

Annexure -I

GRAPH 37 : PERMANENT TOOTH SAMPLE FOR DENTAL PULP STEM CELLS

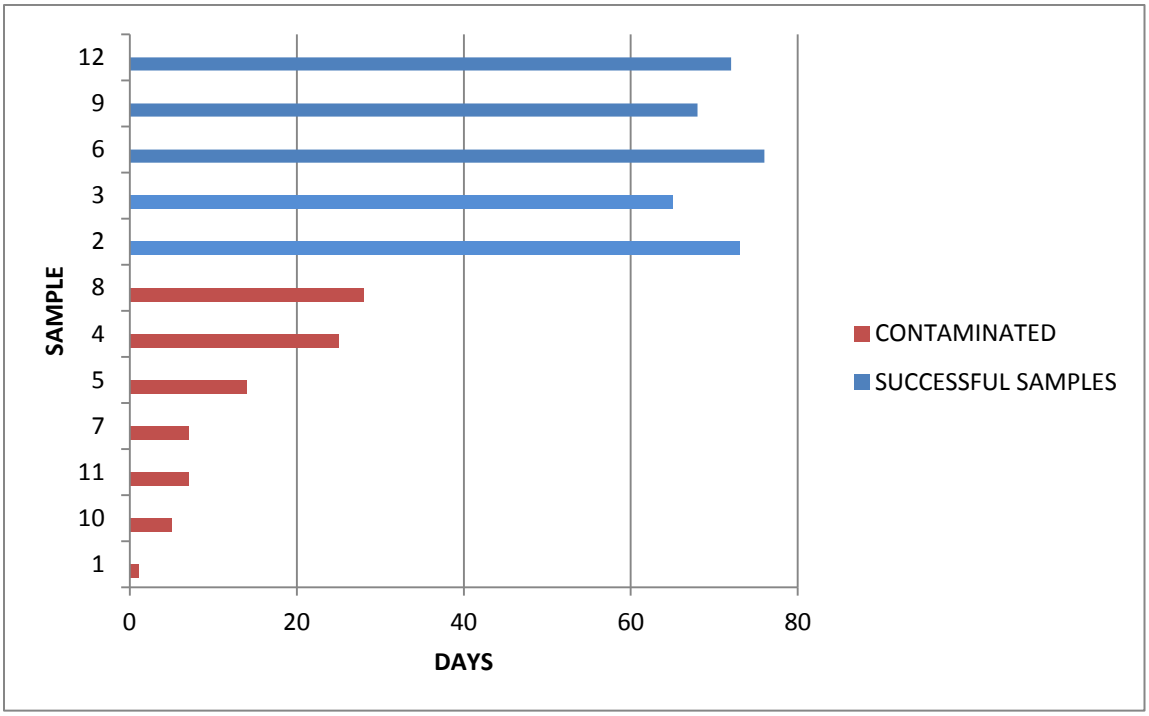


TABLE 46 : PERMANENT TOOTH SAMPLE FOR DENTAL PULP STEM CELLS

SAMPLE NO:	AGE / GENDER	OUTCOME	GROWTH ANALYSIS	SUBPOPULATION ANALYSIS
1	30 Years / female.	Contamination on the first day after plating		
2	23 years / female	Successful*	Done	Done
3	32 years / male	Successful*	Done	Done
4	22 years / female	Contamination on the 25th day, as it was reaching confluence		
5	35 years/ female	Contamination by the end of 2nd week after plating		
6	35 years / female	Successful*	Done	Done
7	28 years / male	Contamination after the first week observation of very few colonies		
8	22years / female	Contamination occurred in the fourth week, there were very few colonies and cells by then		
9	24 years / male	Successful*	Done	Done
10	27 years / female	Contamination present by the end of fifth day		
11	28 years / male	Contamination within the end of first week		
12	22 years / female	Successful*	Done	Done

*-upto fifth passage was done

Annexure - II

GRAPH 38: DECIDUOUS TOOTH SAMPLE FOR STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

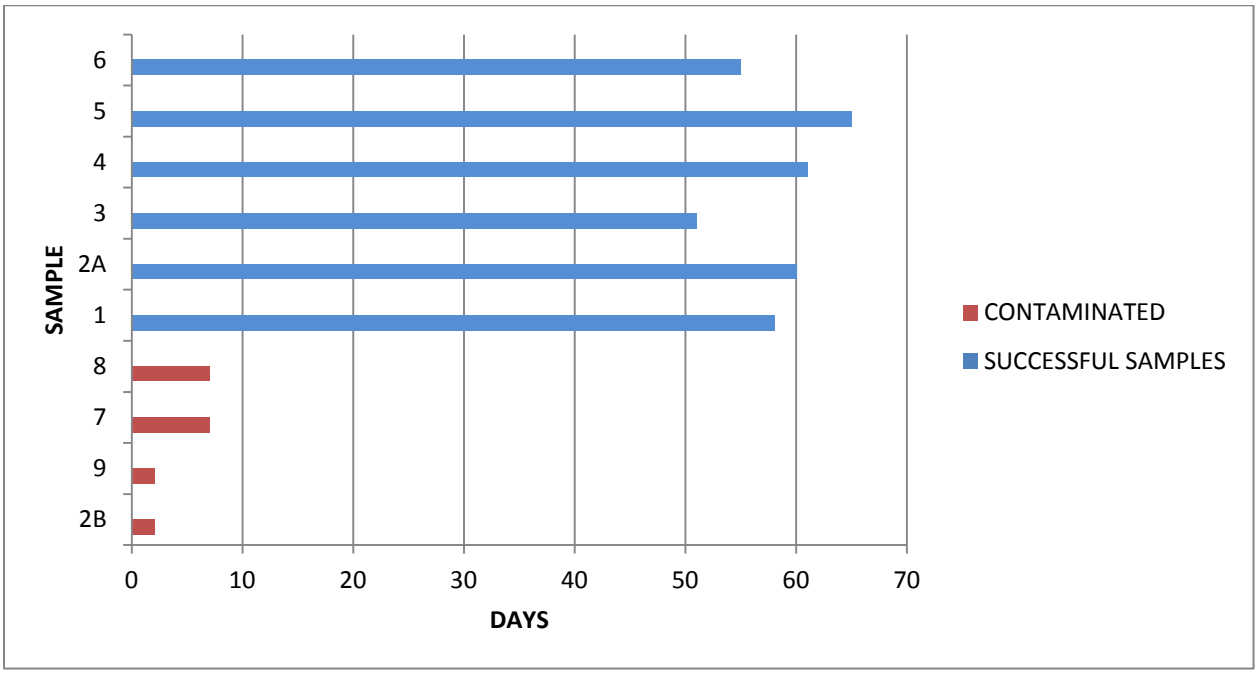


TABLE 47: DECIDUOUS TOOTH SAMPLE FOR STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

SAMPLE NO	DETAILS	OUTCOME	GROWTH ANALYSIS	SUBPOULATION ANALYSIS
1	9 years/male	Successful*	Done	Done
2A	10 years/male	Successful*	Done	Done
2B	10 years/male	Coronal pulp of sample tooth considered as second sample due to caries but contamination was observed in the plate 48 hours after plating		
3	13years/male	Successful*	Done	Done
4	12 years/male	Successful*	Contamination on the fifth day	Contamination on the second day
5	8years/male	Successful*	Contamination on the sixth day	Not done
6	9years/ female	Successful*	Done	Done
7	8years/male	Contamination occurred after seven days of plating		
8	11 years/male	Contamination occurred by the end of first week		
9	11years/male	Contamination observed 48 hours after plating		

*-upto fifth passage was done

Annexure - III Abbrevations

1. MSC – Mesenchymal Stem Cell
2. DPSC – Dental Pulp Stem Cells
3. SHED – Stem cells from Human Exfoliated Deciduous teeth
4. BMMSC – Bone Marrow derived Mesenchymal Stem Cells
5. PDLSC - Periodontal ligament derived Stem Cells
6. SCAP – Stem Cells from Apical Papilla
7. DFPC – Dental Follicle derived Precursor Cells
8. HSC – Haematopoietic Stem Cell
9. αMEM – Alpha modification of Minimal Essential Media
10. DMEM – Dulbecco's Modified Eagle medium
11. RPMI 1640 - Roswell Park Memorial Institute medium. RPMI 1640 uses a bicarbonate buffering system and differs from most mammalian cell culture media in its typical pH 8 formulation.
12. FBS – Fetal Bovine Serum
13. FCS – Fetal Calf Serum
14. CFU – F – Colony Forming Unit of Fibroblast.
15. D-PBS – Dulbecco's Phosphate Buffered Saline
16. PBSA – Phosphate Buffered Saline
17. EDTA – Ethylene-di-amine-tetra-acetic acid
18. BP blade / BP handle – Bard Parker . The surgical manufacturing company.
19. HEPA filter - High-Efficiency Particulate Air. A type of air filter.
20. APES - 3-aminopropyl-triethoxy-silane
21. DPX - distrene , dibutyl phthalate, xylene

22.BSA – Bovine Serum Albumin

23.NIH - National Institute of Health

24. ITS supplement – Insulin , Transferin and Sodium selenite supplement

25. Dkk -1 - Dickkopf- 1

26. PD – Population Doubling

27. DT – Doubling Time

28. FACS – Fluorescent Activated Cell Sorting

29. ICST - International Society for Cellular Therapy

30. DPSC-P1 / P9 Dental Pulp Stem Cells In first passage / 9^{th} passage

Annexure - IV Stem Cell Markers

1. STRO1 - Mesenchymal stem cell marker first identified in bone marrow stromal precursors.
2. CD 106 – Cluster of Differentiation 106 [VCAM - Vascular Cell Adhesion Molecule]
3. PDGF – Platelet Derived Growth Factor
4. HLA – Human Leukocyte Antigen
5. MUC -18, (CD 146) , MCAM – Melanoma Cell Adhesion Molecule. Mesenchymal stem cell maker
6. DSPP – Dentin Sialophosphoprotein
7. Oct-4 - Octamer-binding transcription factor 4. This protein is critically involved in the self-renewal of undifferentiated embryonic stem cells. As such, it is frequently used as a marker for undifferentiated cells. Oct-4 expression must be closely regulated; too much or too little will actually cause differentiation of the cells.
8. Nanog – Transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells. In humans, this protein is determined by the *NANOG* gene.
9. SSEA -3, 4 - Stage Specific Embryonic Antigens. SSEAs were initially recognized by monoclonal antibodies recognizing defined carbohydrate epitopes associated with lacto- and globo-series glycolipids. They are frequently used as markers for stem cell differentiation. SSEA-1 is expressed on murine embryonal carcinoma (EC) cells, embryonic stem (ES) cells, and primordial germ cells. SSEA-3 and SSEA-4 are synthesized during oogenesis and is present on oocyte, zygote, and early cleavage-

stage embryo membranes. Murine SSEA-1 expression decreases with differentiation as SSEA-3 and SSEA-4 expression increase. In contrast, human EC and ES cells express SSEA-3 and SSEA-4, and differentiation is accompanied by an upregulation of SSEA-1 and down-regulation of SSEA-3 and SSEA-4.

10. ABCG2 - ATP-binding cassette superfamily G member 2 is a determinant of the Hoechst-negative phenotype of side population (SP) and found in a wide variety of stem cells, including HSC
11. PSA-NCAM (Polysialic acid-neural cell adhesion molecule): The regulated expression of neural cell adhesion molecule (NCAM) isoforms in the brain is critical for many neural developmental processes. The embryonic form of NCAM, PSA-NCAM, is highly polysialylated and is mainly expressed in the developing nervous system. PSA-NCAM may be related to synaptic rearrangement and plasticity. In the adult, PSA-NCAM expression is restricted to regions that retain plasticity.
12. p75 Neurotrophin R (NTR): p75 NTR, also named low affinity nerve growth factor (NGF) receptor, is a type I transmembrane protein that belongs to the tumor necrosis factor receptor superfamily.
13. Bmi-1: The transcriptional repressor Bmi-1 is one of the polycamb-group proteins, which was discovered as a common oncogene activated in lymphoma9 and later shown to specially regulate hemato-poietic stem cells10. The role of Bmi-1 has also been illustrated in neural stem cells
14. Notch: The Notch pathway has been known to developmental biologists for decades. Its role in control of stem cell proliferation has now been

demonstrated for several cell types including hematopoietic, neural and mammary stem cells.

15. Sonic hedgehog and Wnt: These developmental pathways are also strongly implicated as stem cell regulators.
16. TRA-1-60 & TRA-1-81 - Embryonal stem cell markers. These antibodies recognizes the respective antigens that are expressed on human tetra carcinoma stem cells , human embryonic germ cells and embryonic stem cells. Both the antigens are associated with peri cellular matrix proteogycan.TRA-1-81 immunoprecipitates a 420kD and a 250kD protein and TRA-1-60 immunoprecipitate a 235kD and a 410kD protein from lysates of human tetra carcinoma stem cells.
17. MEPE – Matrix Extracellular Phospho Glycoprotein
18. Nestin - Neural stem cell marker. Nestin is a type VI intermediate filament (IF) protein. These intermediate filament proteins are expressed mostly in nerve cells where they are implicated in the radial growth of the axon
19. Musashi-1 - An evolutionally conserved marker for CNS progenitor cells including neural stem cells
20. Nucleostemin - 62 kDa, three domain nuclear protein that can be found in the nucleoli of embryonic stem cells, CNS stem cells, primitive cells in bone marrow, and cancer cells. It is not found in differentiated cells of most adult tissue. It is suggested to play a role in controlling the cell cycle progression of stem cells and cancer cells.
21. CBFA1 - core-binding factor subunit alpha-1 also known as Runt-related transcription factor 2 (RUNX2) is a protein that in humans is encoded by

the RUNX2 gene. RUNX2 is a key transcription factor associated with osteoblast differentiation.

22. ALP – Alkaline phosphatase plays a role in the organization of actin filament arrays within muscle cells.
23. Osterix – important osteogenic inducer, suppresses chondrogenesis and promotes osteoblastic differentiation at a later stage.
24. Osteocalcin – Osteocalcin belongs to the osteocalcin/matrix Gla protein family and constitutes 1 to 2% of the total bone protein. It is a 49 amino acid single chain vitamin K dependent protein, made by osteoblasts, and is a major component of the non collagenous bone matrix. Post translational modification by a vitamin K dependent carboxylase produces three gamma carboxy glutamic acid residues at

    positions 17, 21 and 24, giving it a high affinity for calcium. It also binds strongly to apatite
25. GAD - Recognizes the gene GAD . This gene encodes one of several forms of glutamic acid decarboxylase, identified as a major autoantigen in insulin-dependent diabetes. The enzyme encoded is responsible for catalyzing the production of gamma aminobutyric acid from L glutamic acid. A pathogenic role for this enzyme has been identified in the human pancreas since it has been identified as an autoantibody and an

    autoreactive T cell target in insulin dependent diabetes. This gene may also play a role in the stiff man syndrome.
26. NeuN - Neuronal nuclear antigen and was first described in 1994 by Mullen et al.

This antigen was proved to bind an antigen expressed only in neuronal nuclei and to a lesser extent the cytoplasm of neuronal cells, and which appeared to work on all

Vertebrates. This unknown antigen was therefore known as NeuN for "Neuronal Nuclei".

27. GFAP – Glial Fibrillary Acidic protein that in humans is encoded by the *GFAP* gene.

    Glial fibrillary acidic protein is an intermediate filament (IF) protein that is expressed by numerous cell types of the central nervous system (CNS) including astrocytes, and ependymal cells.

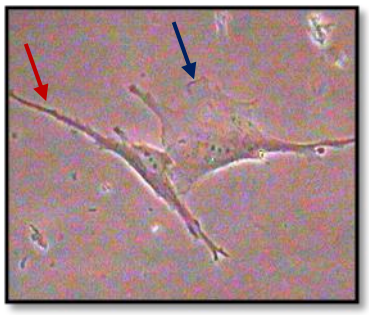
28. CNPase -(2', 3'-cyclic nucleotide 3'-phosphodiesterase catalyzes the in vitro hydrolysis of 2', 3'-cyclic nucleotides to produce 2'nucleotides detects endogenous levels of total CNPase protein…

29. PPAR - Peroxisome Proliferator-Activated Receptor

Annexure - V Terminologies

1. PRIMARY CULTURE – It is that stage of the culture after isolation of the cells but before the first subculture.
2. CONFLUENT – A monolayer of cells in which all cells are in contact with other cells all around their periphery, and no available substrate is left uncovered.
3. SUB CULTURE / PASSAGE - The transfer or subculture of cells from one culture vessel to another; usually, but not necessarily, involves the subdivision of a proliferating cell population, enabling the propagation of a cell line or cell strain.
4. CELL LINES – A propagated culture after the first sub culture
5. GROWTH CURVE – A semi logarithmic plot of the cell number on a logarithmic scale against time on a linear scale, for a proliferating cell culture; usually divided into the lag phase (the phase before growth is initiated), the log phase (the period of exponential growth), and the plateau (a stable cell count achieved when the culture stops growing at a high cell density).
6. SEEDING EFFICIENCY – The percentage of the inoculums that attaches to the substrate within a stated period of time (implying viability, or survival, but not necessarily proliferative capacity).
7. POPULATION DOUBLING TIME – The interval required for a cell population to double at the middle of the logarithmic phase of growth.
8. HAYFLICK LIMIT - or Hayflick Phenomenon is the number of times a normal cell population will divide before it stops, presumably because the telomeres reach a critical length.

ANNEXURE VII – SUBPOPULATION ANALYSIS –Bayreuther . K 1988 [27]

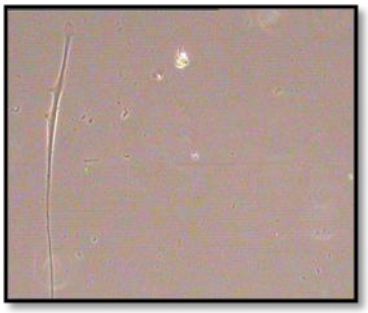


Red arrow – F1 (20 X)

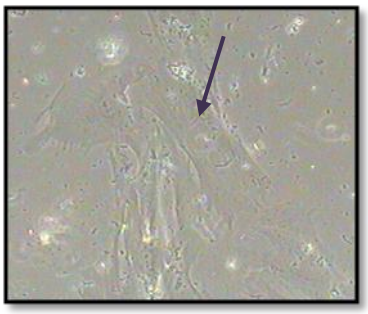
Blue arrow – F3 (20 X)

Red arrow – F1 (10 X)

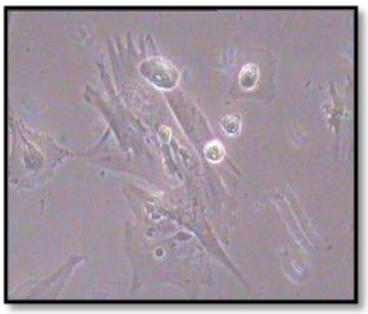
Black arrow – F2 (10 X)



F4 – 10 X



F5 (10 X)



F6 (10 X)

F7 (10 X)

ANNEXURE VI – Subpopulation analysis – Mollenhauer et al 1986 [26]

F1 – 20 X

F2 – 20 X

F 3 – 20 X

Annexure - IX

Abcam plc 330 Cambridge Science Park, Cambridge, CB4 0FL, UK Tel: +44 (0)1223 696000 Fax: +44 (0)1223 771600 www.abcam.com

Mouse monoclonal [1G11B1] to VCAM1 · (ab7219)

Printed on 20 April 2011

Immunogen	Full length protein (Human).
Species Reactivity	Reacts with: Hu
Application notes	The **Abpromise** guarantees that Abcam's products perform as described on the datasheet. If you need assistance to get a product to work as we say it should, please go to www.abcam.com/technical so that we can help you. See www.abcam.com/abpromise for more details. Optimal dilutions/concentrations for use should be determined by the end user. **Recommended dilutions** Flow Cyt: Use at an assay dependent dilution. ICC: Use at an assay dependent dilution. IHC-Fr: Use at an assay dependent dilution. IP: Use at an assay dependent dilution. The antibody is extremely useful for staining of VCAM-1 expressing endothelial cells. It permits staining of *in vitro* cultured cells and frozen tissue sections. The antibody can be used for flow cytometry and immunoprecipitation. *In vitro* cultured cells can be fixed with 1% paraformaldehyde. Tissue sections should be fixed for 10 min in pure acetone followed by incubation for 10 min in chloroform. Incubation with a pretested dilution of the antibody is advised followed by a biotin-conjugated anti-murine Ig and a further incubation with an enzyme (alkaline phosphatase) conjugated streptavidin. For selection of the most useful dilution in a given situation, a test staining with cells or tissue known to express the antigen should be performed. To this end either cultured endothelial cells or a small fresh skin biopsy can be incubated for 8 hours with TNF alpha (1 ng/ml), IL-1 (100 U/ml) or LPS (1 ug/ml) in tissue culture medium at 37°C. Not yet tested in other applications.
Positive Control	Lymph nodes can be used as positive control. As negative control it is advised to use as first antibody a control murine IgG1 antibody.
Target	Information below.
Function	Important in cell-cell recognition. Appears to function in leukocyte-endothelial cell adhesion. Interacts with the beta-1 integrin VLA4 on leukocytes, and mediates both adhesion and signal transduction. The VCAM1/VLA4 interaction may play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation.
Tissue specificity	Expressed on inflammed vascular endothelium, as well as on macrophage-like and dendritic cell types in both normal and inflammed tissue.
Sequence similarities	Contains 7 Ig-like C2-type (immunoglobulin-like) domains.
Domain	Either the first or the fourth Ig-like C2-type domain is required for VLA4-dependent cell adhesion.
Post-translational modifications	Sialoglycoprotein.
Cellular localization	Membrane.
UniProt	Target information above from: UniProt accession P19320
Accession numbers	See online product datasheet at www.abcam.com/ab7219 for the latest database accession numbers.

Abcam plc 330 Cambridge Science Park, Cambridge, CB4 0FL, UK Tel: +44 (0)1223 696000 Fax: +44 (0)1223 771600 www.abcam.com

Clonality	Monoclonal
Clone number	1G11B1
Isotype	IgG1
Purity	IgG fraction
Storage buffer	PBS with 0.2% BSA, 0.1% sodium azide
	This product contains a poisonous or hazardous substance. For material safety datasheet (MSDS) see: Sodium Azide - www.abcam.com/msds.cfm?intMSDSID=3
	Please let us know by return if you are unable to access the Internet and we will either send you a hard copy MSDS or fax the relevant MSDS to you at your request.
Form	Liquid
Concentration	0.10 mg/ml
Storage instructions	Store at +4°C.

All products are "FOR RESEARCH USE ONLY AND ARE NOT INTENDED FOR DIAGNOSTIC OR THERAPEUTIC USE"

After Sales Service (Full terms and conditions can be viewed on www.abcam.com)

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Registered in England, Number: 3509322

Annexure - VIII

Abcam plc 330 Cambridge Science Park, Cambridge, CB4 0FL, UK Tel: +44 (0)1223 696000 Fax: +44 (0)1223 771600 www.abcam.com

Mouse monoclonal [7i35] to STRO1 · (ab102969)

Printed on 31 March 2011

Immunogen	CD34+ bone marrow cells.
Species Reactivity	Reacts with: Hu, Mk
Specificity	ab102969 recognizes a cell surface antigen expressed by bone marrow stromal cells and stromal precursors.
Application notes	The **Abpromise** guarantees that Abcam's products perform as described on the datasheet. If you need assistance to get a product to work as we say it should, please go to www.abcam.com/technical so that we can help you. See www.abcam.com/abpromise for more details. Optimal dilutions/concentrations for use should be determined by the end user. **Recommended dilutions** Flow Cyt: 1/25. ICC/IF: Use at an assay dependent dilution. Not yet tested in other applications.
Product notes	ab102969 binds to approximately 10% of bone marrow mononuclear cells, greater than 95% of which are nucleated erythroid precursors (see references provided).
Clonality	Monoclonal
Clone number	7i35
Isotype	IgM
Purity	Ascites
Storage buffer	Preservative: None Constituents: Ascites
Form	Liquid
Concentration	Concentration not determined.
Storage instructions	Store at +4°C short term (1-2 weeks). Aliquot and store at -20°C (add glycerol to a final volume of 40% for extra stability). Avoid repeated freeze / thaw cycles.

References

This product has been used in:

Simmons PJ & Torok-Storb B Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood***78**:55-62 (1991). **Human.** PubMed: 2070060

Simmons PJ & Torok-Storb B CD34 expression by stromal precursors in normal human adult bone marrow. *Blood***78**:2848-53 (1991). **Human.** PubMed: 1720038

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- Shipping temperature - Our experiments show that the majority of antibodies are stable at room temperature for at least one week, but as a precautionary measure we ship our antibodies in refrigerated recyclable packaging designed to keep our products cool during transit. If the product arrives at ambient temperature, please do not worry, our experiments have shown that activity will not be impaired. Some of our proteins are shipped on dry ice and should arrive frozen.
- Quantity - Liquid products should be centrigued before use. Failure to do so may result in some product remaining in the cap.

Items ordered incorrectly

If items are ordered incorrectly by the customer, Abcam will consider taking them back. Requests for returns must have prior authorization (contact returns@abcam.com) and must be made within 7 days of receipt of the items. Returns are subject to a restocking charge equal to 20% of the order value. Items must be returned in the same or equivalent packaging (i.e. cold and insulated) as originally despatched by Abcam. If you have ordered incorrectly from one of our authorised distributors, please contact them for details of their returns policy.

Annexure - X

Abcam plc 330 Cambridge Science Park, Cambridge, CB4 0FL, UK Tel: +44 (0)1223 696000 Fax: +44 (0)1223 771600 www.abcam.com

Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) · (ab97046)

Printed on 31 March 2011

Species Reactivity	Reacts with: Ms
Specificity	By immunoelectrophoresis and ELISA this antibody reacts specifically with mouse IgG and with light chains common to other mouse immunoglobulins. No antibody was detected against non-immunoglobulin serum proteins.
Application notes	The **Abpromise** guarantees that Abcam's products perform as described on the datasheet. If you need assistance to get a product to work as we say it should, please go to www.abcam.com/technical so that we can help you. See www.abcam.com/abpromise for more details. Optimal dilutions/concentrations for use should be determined by the end user. **Recommended dilutions** ELISA: 1/10000 - 1/10000 (Primary) ICC: Use at an assay dependent dilution. IHC-P: 1/200 - 1/5000. WB: 1/5000 - 1/3000 (Colorimetric). (Chemiluminescent 1/10000 - 1/50000). Predicted molecular weight: 36 kDa. Not yet tested in other applications.
Relevance	Immunoglobulin G (IgG), is one of the most abundant proteins in human serum with normal levels between 8-17 mg/ml in adult blood. IgG is important for our defence against microorganisms and the molecules are produced by B lymphocytes as a part of our adaptive immune response. The IgG molecule has two separate functions; to bind to the pathogen that elicited the response and to recruit other cells and molecules to destroy the antigen. The variability of the IgG pool is generated by somatic recombination and the number of specificities in an individual at a given time point is estimated to be 10^{11} variants.
Accession numbers	See online product datasheet at www.abcam.com/ab97046 for the latest database accession numbers.
Clonality	Polyclonal
Isotype	IgG
Purity	Immunogen affinity purified
Conjugation notes	Molar enzyme/ antibody protein ratio is 4:1
Storage buffer	Preservative: 0.1% Proclin Constituents: 0.2% BSA, PBS
Purification notes	This antibody was isolated by affinity chromatography using antigen coupled to agarose beads and conjugated to Horse Radish Peroxidase (HRP).
Form	Liquid
Conjugation	Horse Radish Peroxidase
Concentration	1.00 mg/ml
Storage instructions	Store at +4°C.
Images	see www.abcam.com/ab97046 for more detail on the image below.

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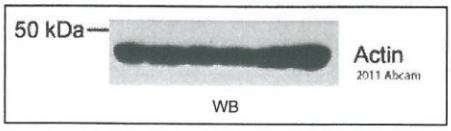

All products are "FOR RESEARCH USE ONLY AND ARE NOT INTENDED FOR DIAGNOSTIC OR THERAPEUTIC USE"

After Sales Service (Full terms and conditions can be viewed on www.abcam.com)

Product condition upon arrival

- Shipping temperature - Our experiments show that the majority of antibodies are stable at room temperature for at least one week, but as a precautionary measure we ship our antibodies in refrigerated recyclable packaging designed to keep our products cool during transit. If the product arrives at ambient temperature, please do not worry, our experiments have shown that activity will not be impaired. Some of our proteins are shipped on dry ice and should arrive frozen.
- Quantity - Liquid products should be centrigued before use. Failure to do so may result in some product remaining in the cap.

Items ordered incorrectly
If items are ordered incorrectly by the customer, Abcam will consider taking them back. Requests for returns must have prior authorization (contact returns@abcam.com) and must be made within 7 days of receipt of the items. Returns are subject to a restocking charge equal to 20% of the order value. Items must be returned in the same or equivalent packaging (i.e. cold and insulated) as originally despatched by Abcam. If you have ordered incorrectly from one of our authorised distributors, please contact them for details of their returns policy. Please note that Abcam does not permit the return of, or offer refunds for incorrectly ordered items in Asia Pacific distributor regions.

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Annexure XI : Institutional Review Board Approval

From,

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi,Chennai

The dissertation topic titled 'Analysis of growth, phenotypic characteristics and expression of STRO1 and CD 106 in Dental Pulp Stem Cells and Stem Cells from Human Exfoliated Deciduous teeth' submitted by Dr. Soundarya .S has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 14th March 2011.

Dr.K.Ranganathan
Secretary,
Ragas , IRB

Dr.S.Ramachandran
Chairman,
Ragas , IRB